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UTILITY PATENT APPLICATION TRANSMITTAL

Inventor(s): Mahajan, et al.

Title: Maize Rad50 Orthologue and Uses Thereof

APPLICATION ELEMENTS

1. Fee Transmittal Form (Submit an original, and a duplicate for fee processing)
2. Specification [Total Pages 68]
(Preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross Reference to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. Drawing(s) (35 USC 113) [Total Sheets]
 - a. Formal
 - b. Informal

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4. Oath or Declaration [Total Pages 3]
a. Newly executed (original or copy)
b. Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]

i. DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5. Incorporation By Reference (useable if Box 4B is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. Microfiche Computer Program (Appendix)

7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
a. Computer Readable Copy
b. Paper Copy (identical to computer copy)
c. Statement verifying identity to above copies

ACCOMPANYING APPLICATION PARTS

8. Assignment Papers (cover sheet & document(s))

9. 37 CFR 3.73(b) Statement Power of Attorney
(where there is an assignee)

10. English Translation Document (if applicable)

11. Information Disclosure Statement (IDS/PTO-1449) Copies of IDS Citations

12. Preliminary Amendment

13. Return Receipt Postcard (MPEP 503) (Should be specifically itemized)

14. Small Entity Statement(s) Statement filed in prior application
Status still proper and desired

Attorney Docket No.: 1116

15. Certified Copy of Priority document(s)

16. Other: Sequence Listing

17. If a **CONTINUING APPLICATION**, check the appropriate box and supply the requisite information: Continuation Divisional Continuation-in-part (CIP) of prior application No: _____.

18. CORRESPONDENCE ADDRESS

Customer Number or Bar Code Label: _____
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or

Correspondence address below

Name: Michael E. Yates

Address: Pioneer Hi-Bred International, Inc.
Corporate Intellectual Property
7100 N.W. 62nd Avenue
P.O. Box 1000

City Johnston State: Iowa Zip Code: 50131-1000

Country: USA Phone: (515) 248-4835 Facsimile: (515) 334-6883

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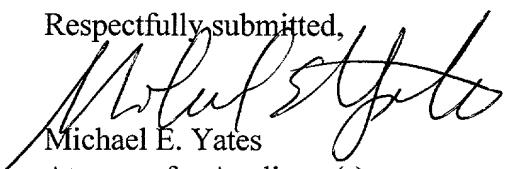
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Respectfully submitted,


Michael E. Yates
Attorney for Applicant(s)
Registration No. 36,063

PIONEER HI-BRED INTERNATIONAL, INC.
Corporate Intellectual Property
7100 N.W. 62nd Avenue
P.O. Box 1000
Johnston, Iowa 50131-1000
Phone: (515) 248-4835
Facsimile: (515) 334-6883

PROVISIONAL PATENT APPLICATION

Maize Rad50 Orthologue and Uses Thereof

Inventors: Pramod B. Mahajan
8029 Brookview Drive
Urbandale, IA 50322
Citizen of the United States of America

Jinrui Shi
6022 Greendale Circle
Apt. 205
Johnston, IA 50131
Citizen of China

Assignee:

PIONEER HI-BRED INTERNATIONAL, INC.
800 Capital Square
400 Locust Street
Des Moines, IA 50309
1-800-247-6803

PATENT

Attorney Docket No. 1116

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Application Serial No. 60/132,575 filed May 5, 1999, which is herein incorporated by reference.

TECHNICAL FIELD

10 The present invention relates generally to plant molecular biology. More specifically, it relates to nucleic acids and methods for modulating their expression in plants.

BACKGROUND OF THE INVENTION

15 The RAD50 gene of *Saccharomyces cerevisiae* plays a crucial role in meiotic recombination as well as DNA repair during vegetative growth (Kupiec, M. and Simchen, G., *Mol. Gen. Genet.* 193: 525-531, 1984). The yeast RAD50 gene encodes a 153 kDa protein (Rad50) that contains an ATP- binding site (Walker -B box or P-loop) in the N-terminal region and exhibits ATP-dependent DNA binding *in vitro* (Raymond, 20 W.E. and Kleckner, N., *Nucleic Acid Res.* 16: 3851-3856, 1993). The Rad50 protein also exhibits two, 250 amino acid segments of heptad-repeat sequence, which form alpha helical coiled coil structures (Alani *et al.*, *Genetics* 122: 47-57, 1989). In yeast, RAD50 deletion mutants show a mitotic hyper-recombinational phenotype. The same mutant exhibits reduced meiotic double strand break formation and recombination (reviewed in 25 Malkova *et al.*, *Genetics* 143: 741-754, 1996 and Jeggo, P., *Radiation Res.* 150: S80-S91, 1998).

Interestingly, similar phenotypes were observed in the deletion mutants for two other yeast genes MRE11 and XRS2, suggesting an involvement of these genes in double-strand break repair and homologous recombination (Malkova *et al.*, *Genetics* 30 143: 741-754, 1996; Jeggo, P., *Radiation Res.* 150: S80-S91, 1998). Subsequently, Jozhuka and Ogawa demonstrated the interaction of yeast Rad50 and Mre11 proteins (Johzuka, K. and Ogawa, H., *Genetics* 139: 1521-1532, 1995). Tsukamoto *et al.*, showed the involvement of yeast RAD50, MER11 and XRS2 as well as HDF1 (yeast

homologue of Ku70) in illegitimate or non-homologous end-joining (Tsukamoto, Y. *et al.*, *Mol. Gen. Genet.* 255: 543-547, 1997).

Recently, mammalian homologues of yeast RAD50 have been cloned and characterized extensively (Kim, K. *et al.*, *J. Biol. Chem.*, 271: 29255-29264, 1996; Dolganov *et al.*, *Mol. Cell. Biol.* 16: 4832-4841, 1996; Carney J.P. *et al.*, *Cell* 93: 477-486, 1998; Trujillo, K.M. *et al.*, *J. Biol. Chem.* 273: 21447-21450, 1998). Similarly, the *Arabidopsis thaliana* chromosome II BACF22D22 region (Accession No. AC006223) has been found to contain an open reading frame which encodes a protein with homology to yeast RAD50 (GI:4263721).

Control of homologous recombination or non-homologous end joining by modulating Rad50 provides the means to modulate the efficiency with which heterologous nucleic acids are incorporated into the genomes of a target plant cell. Control of these processes has important implications in the creation of novel recombinantly engineered crops such as maize. The present invention provides this and other advantages.

SUMMARY OF THE INVENTION

The present invention describes the maize Rad50 protein, which clearly possesses features characteristic of other Rad50 proteins, and has a calculated molecular weight of ~152.5 kDa. The maize Rad50 protein is characterized by the presence of an ATP binding site in the N-terminal region, a second nucleotide binding site in the C-terminal region, putative nuclear localization signals, and heptad-repeats. The presence of extensive leucine zipper structures appears to be another striking feature of the Rad50 proteins. These are also found in the maize Rad50 protein and are indicated in **bold** in Figure 1. The present invention also describes a maize Rad50 polynucleotide sequence. The maize Rad50 orthologue of the present invention was used as a probe to map the maize RAD50 gene(s) to the short arm of chromosome 4.

Generally, it is the object of the present invention to provide nucleic acids and proteins relating to maize Rad50. It is an object of the present invention to provide: 1) antigenic fragments of the proteins of the present invention; 2) transgenic plants comprising the nucleic acids of the present invention; 3) methods for modulating, in a transgenic plant, the expression of the nucleic acids of the present invention.

Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising a member selected from the group consisting of (a) a polynucleotide having a specified sequence identity to a polynucleotide encoding a polypeptide of the present invention; (b) a polynucleotide which is complementary to the polynucleotide of (a); and, 5 (c) a polynucleotide comprising a specified number of contiguous nucleotides from a polynucleotide of (a) or (b). The isolated nucleic acid can be DNA.

In another aspect, the present invention relates to recombinant expression cassettes, comprising a nucleic acid of the present invention operably linked to a promoter.

10 In another aspect, the present invention is directed to a host cell into which has been introduced the recombinant expression cassette.

In a further aspect, the present invention relates to an isolated protein comprising a polypeptide having a specified number of contiguous amino acids encoded by an isolated nucleic acid of the present invention.

15 In another aspect, the present invention relates to an isolated nucleic acid comprising a polynucleotide of specified length which selectively hybridizes under stringent conditions to a polynucleotide of the present invention, or a complement thereof. In some embodiments, the isolated nucleic acid is operably linked to a promoter.

20 In another aspect, the present invention relates to a recombinant expression cassette comprising a nucleic acid amplified from a library as referred to *supra*, wherein the nucleic acid is operably linked to a promoter. In some embodiments, the present invention relates to a host cell transfected with this recombinant expression cassette. In some embodiments, the present invention relates to a protein of the present invention that 25 is produced from this host cell.

In yet another aspect, the present invention relates to a transgenic plant comprising a recombinant expression cassette comprising a plant promoter operably linked to any of the isolated nucleic acids of the present invention. The present invention also provides transgenic seed from the transgenic plant.

30

Definitions

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino

DRAFT - 06/20/2000

acid sequences are written left to right in amino to carboxy orientation, respectively.

Numeric ranges are inclusive of the numbers defining the range and include each integer within the defined range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the

5 IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. Unless otherwise provided for, software, electrical, and electronics terms as used herein are as defined in The New IEEE Standard Dictionary of Electrical and Electronics Terms (5th edition, 1993). The terms defined below are more fully defined by reference to the specification as a whole.

10 By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta

15 Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology: Principles and Applications*, D. H. Persing *et al.*, Ed., American Society for Microbiology, Washington, D.C. (1993). The product of amplification is termed an amplicon.

20 The term "antibody" includes reference to antigen binding forms of antibodies (e.g., Fab, F(ab)₂). The term "antibody" frequently refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of 25 skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bispecific antibodies).

30 The term "antigen" includes reference to a substance to which an antibody can be generated and/or to which the antibody is specifically immunoreactive. The specific immunoreactive sites within the antigen are known as epitopes or antigenic determinants.

DRAFT - 08/2000

These epitopes can be a linear array of monomers in a polymeric composition - such as amino acids in a protein - or consist of or comprise a more complex secondary or tertiary structure. Those of skill will recognize that all immunogens (i.e., substances capable of eliciting an immune response) are antigens; however some antigens, such as haptens, are not immunogens but may be made immunogenic by coupling to a carrier molecule. An antibody immunologically reactive with a particular antigen can be generated *in vivo* or by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors. *See, e.g., Huse et al., Science 246: 1275-1281 (1989); and Ward, et al., Nature 341: 544-546 (1989); and Vaughan et al., Nature Biotech. 14: 309-314 (1996).*

As used herein, "antisense orientation" includes reference to a duplex polynucleotide sequence that is operably linked to a promoter in an orientation where the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited.

As used herein, "chromosomal region" includes reference to a length of a chromosome that may be measured by reference to the linear segment of DNA that it comprises. The chromosomal region can be defined by reference to two unique DNA sequences, i.e., markers.

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein.

For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a

DRAFT 08/28/2018

functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide of the present invention is implicit in each described polypeptide sequence and is within the scope of the present invention.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for its native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) Proteins W.H. Freeman and Company.

By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as are present in some plant, animal, and fungal mitochondria, the bacterium

Mycoplasma capricolum, or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed therein.

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al. Nucl. Acids Res.* 17: 477-498 (1989)). Thus, the maize preferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants are listed in Table 4 of Murray *et al.*, *supra*.

As used herein "full-length sequence" in reference to a specified polynucleotide or its encoded protein means having the entire amino acid sequence of, a native (non-synthetic), endogenous, biologically active form of the specified protein. Methods to determine whether a sequence is full-length are well known in the art including such exemplary techniques as northern or western blots, primer extension, S1 protection, and ribonuclease protection. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997). Comparison to known full-length homologous (orthologous and/or paralogous) sequences can also be used to identify full-length sequences of the present invention. Additionally, consensus sequences typically present at the 5' and 3' untranslated regions of mRNA aid in the identification of a polynucleotide as full-length. For example, the consensus sequence ANNNNAUGG, where the underlined codon represents the N-terminal methionine, aids in determining whether the polynucleotide has a complete 5' end. Consensus sequences at the 3' end, such as polyadenylation sequences, aid in determining whether the polynucleotide has a complete 3' end.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same

species, is substantially modified from its original form by deliberate human intervention.

By "host cell" is meant a cell which contains a vector and supports the replication and/or expression of the vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells. A particularly preferred monocotyledonous host cell is a maize host cell.

The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

By "immunologically reactive conditions" or "immunoreactive conditions" is meant conditions which allow an antibody, reactive to a particular epitope, to bind to that epitope to a detectably greater degree (e.g., at least 2-fold over background) than the antibody binds to substantially any other epitopes in a reaction mixture comprising the particular epitope. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols. See Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

The terms "isolated" refers to material, such as a nucleic acid or a protein, which is: (1) substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment; or (2) if the material is in its natural environment, the material has been synthetically (non-naturally) altered by deliberate human intervention to a composition and/or placed at a location in the cell (e.g., genome or subcellular organelle) not native to a material found in that environment. The alteration to yield the synthetic material can be performed on

the material within or removed from its natural state. For example, a naturally occurring nucleic acid becomes an isolated nucleic acid if it is altered, or if it is transcribed from DNA which has been altered, by means of human intervention performed within the cell from which it originates. See, e.g., Compounds and Methods for Site Directed

5 Mutagenesis in Eukaryotic Cells, Kmiec, U.S. Patent No. 5,565,350; *In Vivo* Homologous Sequence Targeting in Eukaryotic Cells; Zarling *et al.*, PCT/US93/03868. Likewise, a naturally occurring nucleic acid (e.g., a promoter) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome not native to that nucleic acid. Nucleic acids which are "isolated" as defined herein, are also referred to
10 as "heterologous" nucleic acids.

Unless otherwise stated, the term "maize Rad50 nucleic acid" is a nucleic acid of the present invention and means a nucleic acid comprising a polynucleotide of the present invention (a "maize Rad50 polynucleotide") encoding a maize Rad50 polypeptide. A "maize Rad50 gene" is a gene of the present invention and refers to a heterologous
15 genomic form of a full-length maize Rad50 polynucleotide.

As used herein, "localized within the chromosomal region defined by and including" with respect to particular markers includes reference to a contiguous length of a chromosome delimited by and including the stated markers.

As used herein, "marker" includes reference to a locus on a chromosome that
20 serves to identify a unique position on the chromosome. A "polymorphic marker" includes reference to a marker which appears in multiple forms (alleles) such that different forms of the marker, when they are present in a homologous pair, allow transmission of each of the chromosomes of that pair to be followed. A genotype may be defined by use of one or a plurality of markers.

25 As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

30 By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such

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as Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., Vol. 1-3 (1989); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint 5 venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994).

As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence.

10 Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, 15 as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. A particularly 20 preferred plant is *Zea mays*.

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring 25 nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons 30 are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that

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serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells.

5 The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. It
10 will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslational events, including natural processing event and events brought about by human
15 manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Further, this invention contemplates the use of both the methionine-containing and the methionine-less amino terminal variants of the protein of
20 the invention.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells whether nor not its origin is a plant cell. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells such *Agrobacterium* or *Rhizobium*. Examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, or seeds. Such promoters are referred to as "tissue preferred". Promoters which initiate transcription only in certain tissue are referred to as "tissue specific". A "cell type"

specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" or "repressible" promoter is a promoter which is under environmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic 5 conditions or the presence of light. Tissue specific, tissue preferred, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which is active under most environmental conditions.

The term "maize Rad50 polypeptide" is a polypeptide of the present invention 10 and refers to one or more amino acid sequences, in glycosylated or non-glycosylated form. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof. A "maize Rad50 protein" is a protein of the present invention and comprises a maize Rad50 polypeptide.

As used herein "recombinant" includes reference to a cell or vector, that has been 15 modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under-expressed or not expressed at all as a result of deliberate human intervention. The term "recombinant" as used 20 herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements 25 which permit transcription of a particular nucleic acid in a host cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

The term "residue" or "amino acid residue" or "amino acid" are used 30 interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass non-natural analogs.

of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

The term "specifically reactive", includes reference to a binding reaction between an antibody and a protein having an epitope recognized by the antigen binding site of the antibody. This binding reaction is determinative of the presence of a protein having the recognized epitope amongst the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to an analyte having the recognized epitope to a substantially greater degree (e.g., at least 2-fold over background) than to substantially all analytes lacking the epitope which are present in the sample.

Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to the polypeptides of the present invention can be selected from to obtain antibodies specifically reactive with polypeptides of the present invention. The proteins used as immunogens can be in native conformation or denatured so as to provide a linear epitope.

A variety of immunoassay formats may be used to select antibodies specifically reactive with a particular protein (or other analyte). For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine selective reactivity.

The term "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different

circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected

5 (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g.,

10 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M

15 NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

20 Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, *Anal. Biochem.*, 138:267-284 (1984): $T_m = 81.5^{\circ}\text{C} + 16.6(\log M) + 0.41(\%GC) - 0.61(\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1 °C for each 1% of mismatching; thus, T_m , hybridization and/or wash

25 conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic

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strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization

5 and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m).

Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred

10 to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*,

15 Chapter 2, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

As used herein, "transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is

20 passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual

25 propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

30 As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

DRAFT-09-08-09-050

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

5 (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

10 (b) As used herein, "comparison window" includes reference to a contiguous and specified segment of a polynucleotide/polypeptide sequence, wherein the polynucleotide/polypeptide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide/polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

15 Generally, the comparison window is at least 20 contiguous nucleotides/amino acids residues in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide/polypeptide sequence, a gap penalty is typically introduced and is subtracted from the number of matches.

20 Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988);

25 Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, *et al.*, *Nucleic Acids Research* 16: 10881-90 (1988); Huang, *et al.*, *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, *et al.*, *Methods in Molecular Biology* 24: 307-331 (1994).

The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5877 (1993)). One measure of similarity

DRAFT - 05/05/2010

provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance.

BLAST searches assume that proteins can be modeled as random sequences.

5 However, many real proteins comprise regions of nonrandom sequences which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example,

10 the SEG (Wooten and Federhen, *Comput. Chem.*, 17:149-163 (1993)) and XNU (Claverie and States, *Comput. Chem.*, 17:191-201 (1993)) low-complexity filters can be employed alone or in combination.

GAP can also be used to compare a polynucleotide or polypeptide of the present invention with a reference sequence. GAP uses the algorithm of Needleman and Wunsch 15 (*J. Mol. Biol.* 48: 443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit 20 of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide 25 sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can each independently be: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 65 or greater.

30 GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the

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number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters

10 (Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402, 1997; Altschul *et al.*, *J. Mol. Bio.* 215: 403-410, 1990) or to the value obtained using the GAP program using default parameters (see the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA).

15 (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are 20 substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or 25 "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. 30 The scoring of conservative substitutions is calculated, *e.g.*, according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not 5 comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the 10 percentage of sequence identity.

(e) (i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard 15 parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and 20 most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may 25 occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e) (ii) The terms "substantial identity" in the context of a peptide indicates that a 30 peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Optionally, optimal alignment is conducted using the homology alignment algorithm of

Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes.

DETAILED DESCRIPTION OF THE INVENTION

10 Overview

The present invention provides, among other things, compositions and methods for modulating (i.e., increasing or decreasing) the level of polynucleotides and polypeptides of the present invention in plants. In particular, the polynucleotides and polypeptides of the present invention can be expressed temporally or spatially, e.g., at developmental stages, in tissues, and/or in quantities, which are uncharacteristic of non-recombinantly engineered plants. Thus, the present invention provides utility in such exemplary applications as in the control of recombination efficiency or transformation efficiency in plants.

The present invention also provides isolated nucleic acid comprising
20 polynucleotides of sufficient length and complementarity to a gene of the present
invention to use as probes or amplification primers in the detection, quantitation, or
isolation of gene transcripts. For example, isolated nucleic acids of the present invention
can be used as probes in detecting deficiencies in the level of mRNA in screenings for
desired transgenic plants, for detecting mutations in the gene (e.g., substitutions,
25 deletions, or additions), for monitoring upregulation of expression or changes in enzyme
activity in screening assays of compounds, for detection of any number of allelic variants
(polymorphisms), orthologs, or paralogs of the gene, or for site directed mutagenesis in
eukaryotic cells (see, e.g., U.S. Patent No. 5,565,350). The isolated nucleic acids of the
present invention can also be used for recombinant expression of their encoded
30 polypeptides, or for use as immunogens in the preparation and/or screening of
antibodies. The isolated nucleic acids of the present invention can also be employed for
use in sense or antisense suppression of one or more genes of the present invention in a
host cell, tissue, or plant. Attachment of chemical agents which bind, intercalate, cleave

and/or crosslink to the isolated nucleic acids of the present invention can also be used to modulate transcription or translation.

The present invention also provides isolated proteins comprising a polypeptide of the present invention (e.g., preproenzyme, proenzyme, or enzymes). The present invention also provides proteins comprising at least one epitope from a polypeptide of the present invention. The proteins of the present invention can be employed in assays for enzyme agonists or antagonists of enzyme function, or for use as immunogens or antigens to obtain antibodies specifically immunoreactive with a protein of the present invention. Such antibodies can be used in assays for expression levels, for identifying and/or isolating nucleic acids of the present invention from expression libraries, for identification of homologous polypeptides from other species, or for purification of polypeptides of the present invention.

The isolated nucleic acids and polypeptides of the present invention can be used over a broad range of plant types, particularly monocots such as the species of the family *Gramineae* including *Hordeum*, *Secale*, *Triticum*, *Sorghum* (e.g., *S. bicolor*), *Oryza*, *Avena*, and *Zea* (e.g., *Z. mays*). The isolated nucleic acid and proteins of the present invention can also be used in species from the genera: *Cucurbita*, *Rosa*, *Vitis*, *Juglans*, *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Ciahorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Heterocallis*, *Nemesis*, *Pelargonium*, *Panieum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browalia*, *Glycine*, *Pisum*, *Phaseolus*, and *Lolium*.

25

Nucleic Acids

The present invention provides, among other things, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising a polynucleotide of the present invention.

30 A polynucleotide of the present invention is inclusive of:

(a) a polynucleotide encoding a polypeptide of SEQ ID NO: 2 and conservatively modified and polymorphic variants thereof, including exemplary polynucleotides of SEQ ID NO: 1; the polynucleotide sequence of the invention also includes the maize RAD50

Docket No. 16-22950

polynucleotide sequence as contained in a plasmid deposited with American Type Culture Collection (ATCC) and assigned Accession Number 207194.

(b) a polynucleotide which is the product of amplification from a *Zea mays* nucleic acid library using primer pairs which selectively hybridize under stringent conditions to loci within the polynucleotide of SEQ ID NO: 1, or the sequence as contained in ATCC deposit assigned Accession No. 207194, wherein the polynucleotide has substantial sequence identity to the polynucleotide of SEQ ID NO: 1; or the sequence as contained in ATCC deposit assigned Accession No. 207194.

(c) a polynucleotide which selectively hybridizes to a polynucleotide of (a) or (b);

10 (d) a polynucleotide having a specified sequence identity with polynucleotides of (a), (b), or (c);

(e) a polynucleotide encoding a protein having a specified number of contiguous amino acids from a prototype polypeptide, wherein the protein is specifically recognized by antisera elicited by presentation of the protein and wherein the protein does not

15 detectably immunoreact to antisera which has been fully immunosorbed with the protein;

(f) complementary sequences of polynucleotides of (a), (b), (c), (d), or (e); and

(g) a polynucleotide comprising at least a specific number of contiguous nucleotides from a polynucleotide of (a), (b), (c), (d), (e), or (f).

The polynucleotide of SEQ ID NO: 1 is contained in a plasmid deposited with American Type Culture Collection (ATCC) on April 6, 1999 and assigned Accession Number 207194. American Type Culture Collection is located at 10801 University Blvd., Manassas, VA 20110-2209.

The ATCC deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit is provided as a convenience to those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. Section 112. The deposited sequence, as well as the polypeptide encoded by the sequence, is incorporated herein by reference and controls in the event of any conflict, such as a sequencing error, with description in this application.

30 *A. Polynucleotides Encoding A Polypeptide of the Present Invention or Conservatively Modified or Polymorphic Variants Thereof*

As indicated in (a), above, the present invention provides isolated nucleic acids comprising a polynucleotide of the present invention, wherein the polynucleotide encodes

a polypeptide of the present invention, or conservatively modified or polymorphic variants thereof. Accordingly, the present invention includes polynucleotides of SEQ ID NO: 1, and the sequence as contained in ATCC deposit assigned Accession No. 207194, and silent variations of polynucleotides encoding a polypeptide of SEQ ID NO: 2. The

5 present invention further provides isolated nucleic acids comprising polynucleotides encoding conservatively modified variants of a polypeptide of SEQ ID NO: 2.

Conservatively modified variants can be used to generate or select antibodies immunoreactive to the non-variant polypeptide. Additionally, the present invention further provides isolated nucleic acids comprising polynucleotides encoding one or more 10 allelic (polymorphic) variants of polypeptides/polynucleotides. Polymorphic variants are frequently used to follow segregation of chromosomal regions in, for example, marker assisted selection methods for crop improvement.

15 *B. Polynucleotides Amplified from a Zea mays Nucleic Acid Library*

As indicated in (b), above, the present invention provides an isolated nucleic acid comprising a polynucleotide of the present invention, wherein the polynucleotides are amplified from a *Zea mays* nucleic acid library. *Zea mays* lines B73, PHRE1, A632, BMS-P2#10, W23, and Mo17 are known and publicly available. Other publicly known 20 and available maize lines can be obtained from the Maize Genetics Cooperation (Urbana, IL). The nucleic acid library may be a cDNA library, a genomic library, or a library generally constructed from nuclear transcripts at any stage of intron processing. cDNA libraries can be normalized to increase the representation of relatively rare cDNAs. In optional embodiments, the cDNA library is constructed using a full-length cDNA 25 synthesis method. Examples of such methods include Oligo-Capping (Maruyama, K. and Sugano, S. *Gene* 138: 171-174, 1994), Biotinylated CAP Trapper (Carninci, P., Kvan, C., *et al.* *Genomics* 37: 327-336, 1996), and CAP Retention Procedure (Edery, E., Chu, L.L., *et al.* *Molecular and Cellular Biology* 15: 3363-3371, 1995). cDNA synthesis is often catalyzed at 50-55°C to prevent formation of RNA secondary

30 structure. Examples of reverse transcriptases that are relatively stable at these temperatures are SUPERSCRIPT II Reverse Transcriptase (Life Technologies, Inc.), AMV Reverse Transcriptase (Boehringer Mannheim) and RETROAMP Reverse

Transcriptase (Epicentre). Rapidly growing tissues, or rapidly dividing cells are preferably used as mRNA sources.

The present invention also provides subsequences of the polynucleotides of the present invention. A variety of subsequences can be obtained using primers which 5 selectively hybridize under stringent conditions to at least two sites within a polynucleotide of the present invention, or to two sites within the nucleic acid which flank and comprise a polynucleotide of the present invention, or to a site within a polynucleotide of the present invention and a site within the nucleic acid which comprises it. Primers are chosen to selectively hybridize, under stringent hybridization 10 conditions, to a polynucleotide of the present invention. Generally, the primers are complementary to a subsequence of the target nucleic acid which they amplify. As those skilled in the art will appreciate, the sites to which the primer pairs will selectively hybridize are chosen such that a single contiguous nucleic acid can be formed under the desired amplification conditions. In optional embodiments, the primers will be 15 constructed so that they selectively hybridize under stringent conditions to a sequence (or its complement) within the target nucleic acid which comprises the codon encoding the carboxy or amino terminal amino acid residue (i.e., the 3' terminal coding region and 5' terminal coding region, respectively) of the polynucleotides of the present invention. Optionally within these embodiments, the primers will be constructed to selectively 20 hybridize entirely within the coding region of the target polynucleotide of the present invention such that the product of amplification of a cDNA target will consist of the coding region of that cDNA. The primer length in nucleotides is selected from the group of integers consisting of from at least 15 to 50. Thus, the primers can be at least 15, 18, 20, 25, 30, 40, or 50 nucleotides in length. Those of skill will recognize that a 25 lengthened primer sequence can be employed to increase specificity of binding (i.e., annealing) to a target sequence. A non-annealing sequence at the 5'end of a primer (a "tail") can be added, for example, to introduce a cloning site at the terminal ends of the amplicon.

The amplification products can be translated using expression systems well 30 known to those of skill in the art and as discussed, *infra*. The resulting translation products can be confirmed as polypeptides of the present invention by, for example, assaying for the appropriate catalytic activity (e.g., specific activity and/or substrate specificity), or verifying the presence of one or more linear epitopes which are specific

to a polypeptide of the present invention. Methods for protein synthesis from PCR derived templates are known in the art and available commercially. See, e.g., Amersham Life Sciences, Inc, Catalog '97, p.354.

Methods for obtaining 5' and/or 3' ends of a vector insert are well known in the art. See, e.g., RACE (Rapid Amplification of Complementary Ends) as described in Frohman, M. A., in PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, Inc., San Diego), pp. 28-38 (1990); see also, U.S. Pat. No. 5,470,722, and *Current Protocols in Molecular Biology*, Unit 15.6, Ausubel, *et al.*, Eds, Greene Publishing and Wiley-Interscience, New York (1995); Frohman and Martin, *Techniques* 1:165 (1989).

C. Polynucleotides Which Selectively Hybridize to a Polynucleotide of (A) or (B)

As indicated in (c), above, the present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides selectively hybridize, under selective hybridization conditions, to a polynucleotide of sections (A) or (B) as discussed above. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or quantifying nucleic acids comprising the polynucleotides of (A) or (B). For example, polynucleotides of the present invention can be used to identify, isolate, or amplify partial or full-length clones in a deposited library.

In some embodiments, the polynucleotides are genomic or cDNA sequences isolated or otherwise complementary to a cDNA from a dicot or monocot nucleic acid library. Exemplary species of monocots and dicots include, but are not limited to: corn, canola, soybean, cotton, wheat, sorghum, sunflower, oats, sugar cane, millet, barley, and rice. Optionally, the cDNA library comprises at least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low stringency hybridization conditions are typically, but not exclusively, employed with sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

D. Polynucleotides Having a Specific Sequence Identity with the Polynucleotides of (A), (B) or (C)

As indicated in (d), above, the present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides have a 5 specified identity at the nucleotide level to a polynucleotide as disclosed above in sections (A), (B), or (C), above. The percentage of identity to a reference sequence is at least 60% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 60 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 70%, 75%, 80%, 81%, 10 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

Optionally, the polynucleotides of this embodiment will encode a polypeptide that will share an epitope with a polypeptide encoded by the polynucleotides of sections (A), (B), or (C). Thus, these polynucleotides encode a first polypeptide which elicits 15 production of antisera comprising antibodies which are specifically reactive to a second polypeptide encoded by a polynucleotide of (A), (B), or (C). However, the first polypeptide does not bind to antisera raised against itself when the antisera has been fully immunosorbed with the first polypeptide. Hence, the polynucleotides of this embodiment can be used to generate antibodies for use in, for example, the screening of 20 expression libraries for nucleic acids comprising polynucleotides of (A), (B), or (C), or for purification of, or in immunoassays for, polypeptides encoded by the polynucleotides of (A), (B), or (C). The polynucleotides of this embodiment embrace nucleic acid sequences which can be employed for selective hybridization to a polynucleotide encoding a polypeptide of the present invention.

25 Screening polypeptides for specific binding to antisera can be conveniently achieved using peptide display libraries. This method involves the screening of large collections of peptides for individual members having the desired function or structure. Antibody screening of peptide display libraries is well known in the art. The displayed peptide sequences can be from 3 to 5000 or more amino acids in length, frequently from 30 5-100 amino acids long, and often from about 8 to 15 amino acids long. In addition to direct chemical synthetic methods for generating peptide libraries, several recombinant DNA methods have been described. One type involves the display of a peptide sequence on the surface of a bacteriophage or cell. Each bacteriophage or cell contains

the nucleotide sequence encoding the particular displayed peptide sequence. Such methods are described in PCT patent publication Nos. 91/17271, 91/18980, 91/19818, and 93/08278. Other systems for generating libraries of peptides have aspects of both *in vitro* chemical synthesis and recombinant methods. See, PCT Patent publication Nos. 5 92/05258, 92/14843, and 96/19256. See also, U.S. Patent Nos. 5,658,754; and 5,643,768. Peptide display libraries, vectors, and screening kits are commercially available from such suppliers as Invitrogen (Carlsbad, CA).

E. Polynucleotides Encoding a Protein Having a Subsequence from a Prototype

10 *Polypeptide and is Cross-Reactive to the Prototype Polypeptide*

As indicated in (e), above, the present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides encode a protein having a subsequence of contiguous amino acids from a prototype polypeptide of the present invention such as are provided in (a), above. The length of contiguous 15 amino acids from the prototype polypeptide is selected from the group of integers consisting of from at least 10 to the number of amino acids within the prototype sequence. Thus, for example, the polynucleotide can encode a polypeptide having a subsequence having at least 10, 15, 20, 25, 30, 35, 40, 45, or 50, contiguous amino acids from the prototype polypeptide. Further, the number of such subsequences 20 encoded by a polynucleotide of the instant embodiment can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5. The subsequences can be separated by any integer of nucleotides from 1 to the number of nucleotides in the sequence such as at least 5, 10, 15, 25, 50, 100, or 200 nucleotides.

The proteins encoded by polynucleotides of this embodiment, when presented as 25 an immunogen, elicit the production of polyclonal antibodies which specifically bind to a prototype polypeptide such as but not limited to, a polypeptide encoded by the polynucleotide of (a) or (b), above. Generally, however, a protein encoded by a polynucleotide of this embodiment does not bind to antisera raised against the prototype polypeptide when the antisera has been fully immunosorbed with the prototype 30 polypeptide. Methods of making and assaying for antibody binding specificity/affinity are well known in the art. Exemplary immunoassay formats include ELISA, competitive immunoassays, radioimmunoassays, Western blots, indirect immunofluorescent assays and the like.

In a preferred assay method, fully immunosorbed and pooled antisera which is elicited to the prototype polypeptide can be used in a competitive binding assay to test the protein. The concentration of the prototype polypeptide required to inhibit 50% of the binding of the antisera to the prototype polypeptide is determined. If the amount of the protein required to inhibit binding is less than twice the amount of the prototype protein, then the protein is said to specifically bind to the antisera elicited to the immunogen. Accordingly, the proteins of the present invention embrace allelic variants, conservatively modified variants, and minor recombinant modifications to a prototype polypeptide.

10 A polynucleotide of the present invention optionally encodes a protein having a molecular weight as the non-glycosylated protein within 20% of the molecular weight of the full-length non-glycosylated polypeptides of the present invention. Molecular weight can be readily determined by SDS-PAGE under reducing conditions. Optionally, the molecular weight is within 15% of a full length polypeptide of the present invention, 15 more preferably within 10% or 5%, and most preferably within 3%, 2%, or 1% of a full length polypeptide of the present invention.

Optionally, the polynucleotides of this embodiment will encode a protein having a specific enzymatic activity at least 50%, 60%, 80%, or 90% of a cellular extract comprising the native, endogenous full-length polypeptide of the present invention.

20 Further, the proteins encoded by polynucleotides of this embodiment will optionally have a substantially similar affinity constant (K_m) and/or catalytic activity (i.e., the microscopic rate constant, k_{cat}) as the native endogenous, full-length protein. Those of skill in the art will recognize that k_{cat}/K_m value determines the specificity for competing substrates and is often referred to as the specificity constant. Proteins of this
25 embodiment can have a k_{cat}/K_m value at least 10% of a full-length polypeptide of the present invention as determined using the endogenous substrate of that polypeptide. Optionally, the k_{cat}/K_m value will be at least 20%, 30%, 40%, 50%, and most preferably at least 60%, 70%, 80%, 90%, or 95% the k_{cat}/K_m value of the full-length polypeptide of the present invention. Determination of k_{cat} , K_m , and k_{cat}/K_m can be determined by any
30 number of means well known to those of skill in the art. For example, the initial rates (i.e., the first 5% or less of the reaction) can be determined using rapid mixing and sampling techniques (e.g., continuous-flow, stopped-flow, or rapid quenching techniques), flash photolysis, or relaxation methods (e.g., temperature jumps) in

conjunction with such exemplary methods of measuring as spectrophotometry, spectrofluorimetry, nuclear magnetic resonance, or radioactive procedures. Kinetic values are conveniently obtained using a Lineweaver-Burk or Eadie-Hofstee plot.

5 *F. Polynucleotides Complementary to the Polynucleotides of (A)-(E)*

As indicated in (f), above, the present invention provides isolated nucleic acids comprising polynucleotides complementary to the polynucleotides of paragraphs A-E, above. As those of skill in the art will recognize, complementary sequences base-pair throughout the entirety of their length with the polynucleotides of sections (A)-(E) (i.e.,

10 have 100% sequence identity over their entire length). Complementary bases associate through hydrogen bonding in double stranded nucleic acids. For example, the following base pairs are complementary: guanine and cytosine; adenine and thymine; and adenine and uracil.

15 *G. Polynucleotides Which are Subsequences of the Polynucleotides of (A)-(F)*

As indicated in (g), above, the present invention provides isolated nucleic acids comprising polynucleotides which comprise at least 15 contiguous bases from the polynucleotides of sections (A) through (F) as discussed above. The length of the polynucleotide is given as an integer selected from the group consisting of from at least

20 15 to the length of the nucleic acid sequence from which the polynucleotide is a subsequence of. Thus, for example, polynucleotides of the present invention are inclusive of polynucleotides comprising at least 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 contiguous nucleotides in length from the polynucleotides of (A)-(F). Optionally, the number of such subsequences encoded by a

25 polynucleotide of the instant embodiment can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5. The subsequences can be separated by any integer of nucleotides from 1 to the number of nucleotides in the sequence such as at least 5, 10, 15, 25, 50, 100, or 200 nucleotides.

The subsequences of the present invention can comprise structural characteristics of the sequence from which it is derived. Alternatively, the subsequences can lack certain structural characteristics of the larger sequence from which it is derived such as a poly (A) tail. Optionally, a subsequence from a polynucleotide encoding a polypeptide having at least one linear epitope in common with a prototype polypeptide sequence as

provided in (a), above, may encode an epitope in common with the prototype sequence. Alternatively, the subsequence may not encode an epitope in common with the prototype sequence but can be used to isolate the larger sequence by, for example, nucleic acid hybridization with the sequence from which it's derived. Subsequences can be used to 5 modulate or detect gene expression by introducing into the subsequences compounds which bind, intercalate, cleave and/or crosslink to nucleic acids. Exemplary compounds include acridine, psoralen, phenanthroline, naphthoquinone, daunomycin or chloroethylaminoaryl conjugates.

10 **Construction of Nucleic Acids**

The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified, or otherwise constructed from a monocot. In preferred embodiments the monocot is *Zea* 15 *mays*.

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in 20 the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention. A polynucleotide of the present invention can be attached to a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression 25 sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters, 30 and linkers is well known and extensively described in the art. For a description of various nucleic acids see, for example, Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA); and, Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, IL).

A. Recombinant Methods for Constructing Nucleic Acids

The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or a hybrid thereof, can be obtained from plant biological sources using any number of cloning methodologies known to those of skill in the art. In some 5 embodiments, oligonucleotide probes which selectively hybridize, under stringent conditions, to the polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library. While isolation of RNA, and construction of cDNA and genomic libraries is well known to those of ordinary skill in the art, the following highlights some of the methods employed.

10

A1. mRNA Isolation and Purification

Total RNA from plant cells comprises such nucleic acids as mitochondrial RNA, chloroplastic RNA, rRNA, tRNA, hnRNA and mRNA. Total RNA preparation typically involves lysis of cells and removal of organelles and proteins, followed by 15 precipitation of nucleic acids. Extraction of total RNA from plant cells can be accomplished by a variety of means. Frequently, extraction buffers include a strong detergent such as SDS and an organic denaturant such as guanidinium isothiocyanate, guanidine hydrochloride or phenol. Following total RNA isolation, poly(A)⁺ mRNA is typically purified from the remainder RNA using oligo(dT) cellulose. Exemplary total 20 RNA and mRNA isolation protocols are described in *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). Total RNA and mRNA isolation kits are commercially available from vendors such as Stratagene (La Jolla, CA), Clonetech (Palo Alto, CA), Pharmacia 25 (Piscataway, NJ), and 5'-3' (Paoli Inc., PA). See also, U.S. Patent Nos. 5,614,391; and, 5,459,253. The mRNA can be fractionated into populations with size ranges of about 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 kb. The cDNA synthesized for each of these fractions can be size selected to the same size range as its mRNA prior to vector insertion. This method helps eliminate truncated cDNA formed by incompletely reverse 30 transcribed mRNA.

A2. Construction of a cDNA Library

Construction of a cDNA library generally entails five steps. First, first strand cDNA synthesis is initiated from a poly(A)⁺ mRNA template using a poly(dT) primer or

random hexanucleotides. Second, the resultant RNA-DNA hybrid is converted into double stranded cDNA, typically by reaction with a combination of RNase H and DNA polymerase I (or Klenow fragment). Third, the termini of the double stranded cDNA are ligated to adaptors. Ligation of the adaptors can produce cohesive ends for cloning.

5 Fourth, size selection of the double stranded cDNA eliminates excess adaptors and primer fragments, and eliminates partial cDNA molecules due to degradation of mRNAs or the failure of reverse transcriptase to synthesize complete first strands. Fifth, the cDNAs are ligated into cloning vectors and packaged. cDNA synthesis protocols are well known to the skilled artisan and are described in such standard references as: *Plant*
 10 *Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). cDNA synthesis kits are available from a variety of commercial vendors such as Stratagene or Pharmacia.

15 A number of cDNA synthesis protocols have been described which provide substantially pure full-length cDNA libraries. Substantially pure full-length cDNA libraries are constructed to comprise at least 90%, and more preferably at least 93% or 95% full-length inserts amongst clones containing inserts. The length of insert in such libraries can be from 0 to 8, 9, 10, 11, 12, 13, or more kilobase pairs. Vectors to accommodate inserts of these sizes are known in the art and available commercially. See,
 20 e.g., Stratagene's lambda ZAP Express (cDNA cloning vector with 0 to 12 kb cloning capacity).

25 An exemplary method of constructing a greater than 95% pure full-length cDNA library is described by Carninci *et al.*, *Genomics*, 37:327-336 (1996). In that protocol, the cap-structure of eukaryotic mRNA is chemically labeled with biotin. By using streptavidin-coated magnetic beads, only the full-length first-strand cDNA/mRNA hybrids are selectively recovered after RNase I treatment. The method provides a high yield library with an unbiased representation of the starting mRNA population. Other methods for producing full-length libraries are known in the art. See, e.g., Edery *et al.*, *Mol. Cell Biol.*, 15(6):3363-3371 (1995); and, PCT Application WO 96/34981.

30

A3. Normalized or Subtracted cDNA Libraries

A non-normalized cDNA library represents the mRNA population of the tissue it was made from. Since unique clones are out-numbered by clones derived from highly

expressed genes their isolation can be laborious. Normalization of a cDNA library is the process of creating a library in which each clone is more equally represented.

A number of approaches to normalize cDNA libraries are known in the art. One approach is based on hybridization to genomic DNA. The frequency of each hybridized 5 cDNA in the resulting normalized library would be proportional to that of each corresponding gene in the genomic DNA. Another approach is based on kinetics. If cDNA reannealing follows second-order kinetics, rarer species anneal less rapidly and the remaining single-stranded fraction of cDNA becomes progressively more normalized during the course of the hybridization. Specific loss of any species of cDNA, regardless 10 of its abundance, does not occur at any Cot value. Construction of normalized libraries is described in Ko, *Nucl. Acids. Res.*, 18(19):5705-5711 (1990); Patanjali *et al.*, *Proc. Natl. Acad. U.S.A.*, 88:1943-1947 (1991); U.S. Patents 5,482,685, and 5,637,685. In an exemplary method described by Soares *et al.*, normalization resulted in reduction of 15 the abundance of clones from a range of four orders of magnitude to a narrow range of only 1 order of magnitude. *Proc. Natl. Acad. Sci. USA*, 91:9228-9232 (1994).

Subtracted cDNA libraries are another means to increase the proportion of less abundant cDNA species. In this procedure, cDNA prepared from one pool of mRNA is depleted of sequences present in a second pool of mRNA by hybridization. The cDNA:mRNA hybrids are removed and the remaining un-hybridized cDNA pool is 20 enriched for sequences unique to that pool. See, Foote *et al.* in, *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); Kho and Zarbl, *Technique*, 3(2):58-63 (1991); Sive and St. John, *Nucl. Acids Res.*, 16(22):10937 25 (1988); *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); and, Swaroop *et al.*, *Nucl. Acids Res.*, 19(8):1954 (1991). cDNA subtraction kits are commercially available. See, e.g., PCR-Select (Clontech, Palo Alto, CA).

A4. Construction of a Genomic Library

To construct genomic libraries, large segments of genomic DNA are generated by 30 fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. Methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate molecular biological techniques and

instructions sufficient to direct persons of skill through many construction, cloning, and screening methodologies are found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Vols. 1-3 (1989), Methods in Enzymology, Vol. 152: *Guide to Molecular Cloning Techniques*, Berger and Kimmel, Eds., San Diego: Academic Press, Inc. (1987), *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997). Kits for construction of genomic libraries are also commercially available.

10

A5. Nucleic Acid Screening and Isolation Methods

The cDNA or genomic library can be screened using a probe based upon the sequence of a polynucleotide of the present invention such as those disclosed herein. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate 15 homologous genes in the same or different plant species. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The 20 degree of stringency can be controlled by temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%. The degree of complementarity (sequence identity) required for detectable 25 binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor sequence variations in the probes and primers may be compensated for by reducing the stringency of the hybridization and/or wash medium.

The nucleic acids of interest can also be amplified from nucleic acid samples 30 using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of polynucleotides of the present invention and related genes directly from genomic DNA or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic

acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and

5 Ausubel, as well as Mullis *et al.*, U.S. Patent No. 4,683,202 (1987); and, *PCR Protocols A Guide to Methods and Applications*, Innis *et al.*, Eds., Academic Press Inc., San Diego, CA (1990). Commercially available kits for genomic PCR amplification are known in the art. See, e.g., Advantage-GC Genomic PCR Kit (Clontech). The T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

10 PCR-based screening methods have also been described. Wilfinger *et al.* describe a PCR-based method in which the longest cDNA is identified in the first step so that incomplete clones can be eliminated from study. *BioTechniques*, 22(3): 481-486 (1997). In that method, a primer pair is synthesized with one primer annealing to the 5' end of the sense strand of the desired cDNA and the other primer to the vector. Clones 15 are pooled to allow large-scale screening. By this procedure, the longest possible clone is identified amongst candidate clones. Further, the PCR product is used solely as a diagnostic for the presence of the desired cDNA and does not utilize the PCR product itself. Such methods are particularly effective in combination with a full-length cDNA construction methodology, above.

20

B. Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.* 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers, *Tetra. Letts.* 22(20): 1859-1862 (1981), e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter *et al.*, *Nucleic Acids Res.*, 12: 6159-6168 (1984); and, the solid support method of U.S. Patent 25 30 No. 4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of

DNA is best employed for sequences of about 100 bases or less, longer sequences may be obtained by the ligation of shorter sequences.

5 Recombinant Expression Cassettes

The present invention further provides recombinant expression cassettes comprising a nucleic acid of the present invention. A nucleic acid sequence coding for the desired polypeptide of the present invention, for example a cDNA or a genomic sequence encoding a full length polypeptide of the present invention, can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

15 For example, plant expression vectors may include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

20

A plant promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the ubiquitin 1 promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the *Nos* promoter, the pEmu promoter, the rubisco promoter, the GRP1-8 promoter, and other transcription initiation regions from various plant genes known to those of skill. One exemplary promoter is the ubiquitin promoter, which can be used to drive expression of the present invention in maize embryos or embryogenic callus.

Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Environmental conditions that may effect transcription by 5 inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. Examples of inducible promoters are the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, and the PPDK promoter which is inducible by light.

Examples of promoters under developmental control include promoters that 10 initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. Exemplary promoters include the anther specific promoter 5126 (U.S. Patent Nos. 5,689,049 and 5,689,051), glob-1 promoter, and gamma-zein promoter. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in 15 certain locations.

Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the nucleic acids of the present invention. These 20 promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter concentration and/or composition of the proteins of the present invention in a desired tissue. Thus, in some embodiments, the nucleic acid construct will comprise a promoter functional in a plant cell, such as in *Zea mays*, operably linked to a polynucleotide of the present invention. Promoters useful in these embodiments include the endogenous promoters driving 25 expression of a polypeptide of the present invention.

In some embodiments, isolated nucleic acids which serve as promoter or enhancer elements can be introduced in the appropriate position (generally upstream) of a non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example, 30 endogenous promoters can be altered *in vivo* by mutation, deletion, and/or substitution (see, Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868), or isolated promoters can be introduced into a plant cell in the proper orientation and distance from a gene of the present invention so as to control the expression of the gene. Gene expression can be modulated under conditions suitable for plant growth so as to alter the

total concentration and/or alter the composition of the polypeptides of the present invention in plant cell. Thus, the present invention provides compositions, and methods for making, heterologous promoters and/or enhancers operably linked to a native, endogenous (i.e., non-heterologous) form of a polynucleotide of the present invention.

5 Methods for identifying promoters with a particular expression pattern, in terms of, e.g., tissue type, cell type, stage of development, and/or environmental conditions, are well known in the art. See, e.g., *The Maize Handbook*, Chapters 114-115, Freeling and Walbot, Eds., Springer, New York (1994); *Corn and Corn Improvement*, 3rd edition, Chapter 6, Sprague and Dudley, Eds., American Society of Agronomy, Madison, 10 Wisconsin (1988). A typical step in promoter isolation methods is identification of gene products that are expressed with some degree of specificity in the target tissue. Amongst the range of methodologies are: differential hybridization to cDNA libraries; subtractive hybridization; differential display; differential 2-D protein gel electrophoresis; DNA probe arrays; and isolation of proteins known to be expressed with some specificity in 15 the target tissue. Such methods are well known to those of skill in the art. Commercially available products for identifying promoters are known in the art such as Clontech's (Palo Alto, CA) Universal GenomeWalker Kit.

For the protein-based methods, it is helpful to obtain the amino acid sequence for at least a portion of the identified protein, and then to use the protein sequence as the 20 basis for preparing a nucleic acid that can be used as a probe to identify either genomic DNA directly, or preferably, to identify a cDNA clone from a library prepared from the target tissue. Once such a cDNA clone has been identified, that sequence can be used to identify the sequence at the 5' end of the transcript of the indicated gene. For differential hybridization, subtractive hybridization and differential display, the nucleic 25 acid sequence identified as enriched in the target tissue is used to identify the sequence at the 5' end of the transcript of the indicated gene. Once such sequences are identified, starting either from protein sequences or nucleic acid sequences, any of these sequences identified as being from the gene transcript can be used to screen a genomic library prepared from the target organism. Methods for identifying and confirming the 30 transcriptional start site are well known in the art.

In the process of isolating promoters expressed under particular environmental conditions or stresses, or in specific tissues, or at particular developmental stages, a number of genes are identified that are expressed under the desired circumstances, in the

desired tissue, or at the desired stage. Further analysis will reveal expression of each particular gene in one or more other tissues of the plant. One can identify a promoter with activity in the desired tissue or condition but that does not have activity in any other common tissue.

5 To identify the promoter sequence, the 5' portions of the clones described here are analyzed for sequences characteristic of promoter sequences. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually an AT-rich stretch of 5-10 bp located approximately 20 to 40 base pairs upstream of the transcription start site. Identification of the TATA box is well known in the art.

10 For example, one way to predict the location of this element is to identify the transcription start site using standard RNA-mapping techniques such as primer extension, S1 analysis, and/or RNase protection. To confirm the presence of the AT-rich sequence, a structure-function analysis can be performed involving mutagenesis of the putative region and quantification of the mutation's effect on expression of a linked downstream 15 reporter gene. See, e.g., *The Maize Handbook*, Chapter 114, Freeling and Walbot, Eds., Springer, New York, (1994).

In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element (i.e., the CAAT box) with a series of adenines surrounding the trinucleotide G (or T) N G. J. Messing *et al.*, in *Genetic Engineering in Plants*, 20 Kosage, Meredith and Hollaender, Eds., pp. 221-227 (1983). In maize, there is no well conserved CAAT box but there are several short, conserved protein-binding motifs upstream of the TATA box. These include motifs for the trans-acting transcription factors involved in light regulation, anaerobic induction, hormonal regulation, or anthocyanin biosynthesis, as appropriate for each gene.

25 Once promoter and/or gene sequences are known, a region of suitable size is selected from the genomic DNA that is 5' to the transcriptional start, or the translational start site, and such sequences are then linked to a coding sequence. If the transcriptional start site is used as the point of fusion, any of a number of possible 5' untranslated regions can be used in between the transcriptional start site and the partial coding 30 sequence. If the translational start site at the 3' end of the specific promoter is used, then it is linked directly to the methionine start codon of a coding sequence.

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The

polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene.

5 An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold. Buchman and Berg,

10 *Mol. Cell Biol.* 8: 4395-4405 (1988); Callis *et al.*, *Genes Dev.* 1: 1183-1200 (1987). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1 intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994).

15 The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes including genes coding for resistance to the antibiotic spectinomycin (e.g., the *aada* gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin

20 resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resistance to herbicides which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such

25 resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS gene encodes resistance to the herbicide

30 chlorsulfuron.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, *Meth. In Enzymol.*, 153:253-277

(1987). These vectors are plant integrating vectors in that on transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA).

A polynucleotide of the present invention can be expressed in either sense or anti-sense orientation as desired. It will be appreciated that control of gene expression in either sense or anti-sense orientation can have a direct impact on the observable plant characteristics. Antisense technology can be conveniently used to inhibit gene expression in plants. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been shown that antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy *et al.*, Proc. Nat'l. Acad. Sci. (USA) 85: 8805-8809 (1988); and Hiatt *et al.*, U.S. Patent No. 4,801,340.

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli *et al.*, *The Plant Cell* 2: 279-289 (1990) and U.S. Patent No. 5,034,323.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of plant genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff *et al.*, *Nature* 334: 585-591 (1988).

A variety of cross-linking agents, alkylating agents and radical generating species as pendant groups on polynucleotides of the present invention can be used to bind, label,

detect, and/or cleave nucleic acids. For example, Vlassov, V. V., *et al.*, *Nucleic Acids Res* (1986) 14:4065-4076, describe covalent bonding of a single-stranded DNA fragment with alkylating derivatives of nucleotides complementary to target sequences. A report of similar work by the same group is that by Knorre, D. G., *et al.*, *Biochimie* (1985)

5 67:785-789. Iverson and Dervan also showed sequence-specific cleavage of single-stranded DNA mediated by incorporation of a modified nucleotide which was capable of activating cleavage (*J Am Chem Soc* (1987) 109:1241-1243). Meyer, R. B., *et al.*, *J Am Chem Soc* (1989) 111:8517-8519, effect covalent crosslinking to a target nucleotide using an alkylating agent complementary to the single-stranded target nucleotide sequence. A
10 photoactivated crosslinking to single-stranded oligonucleotides mediated by psoralen was disclosed by Lee, B. L., *et al.*, *Biochemistry* (1988) 27:3197-3203. Use of crosslinking in triple-helix forming probes was also disclosed by Home, *et al.*, *J Am Chem Soc* (1990) 112:2435-2437. Use of N4, N4-ethanocytosine as an alkylating agent to crosslink to single-stranded oligonucleotides has also been described by Webb and Matteucci, *J
15 Am Chem Soc* (1986) 108:2764-2765; *Nucleic Acids Res* (1986) 14:7661-7674; Feteritz *et al.*, *J. Am. Chem. Soc.* 113:4000 (1991). Various compounds to bind, detect, label, and/or cleave nucleic acids are known in the art. See, for example, U.S. Patent Nos. 5,543,507; 5,672,593; 5,484,908; 5,256,648; and, 5,681941.

20 **Proteins**

The isolated proteins of the present invention comprise a polypeptide having at least 10 amino acids encoded by any one of the polynucleotides of the present invention as discussed more fully, above, or polypeptides which are conservatively modified variants thereof. The proteins of the present invention or variants thereof can comprise 25 any number of contiguous amino acid residues from a polypeptide of the present invention, wherein that number is selected from the group of integers consisting of from 10 to the number of residues in a full-length polypeptide of the present invention. Optionally, this subsequence of contiguous amino acids is at least 15, 20, 25, 30, 35, or 40 amino acids in length, often at least 50, 60, 70, 80, or 90 amino acids in length.
30 Further, the number of such subsequences can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5.

The present invention further provides a protein comprising a polypeptide having a specified sequence identity with a polypeptide of the present invention. The percentage of

sequence identity is an integer selected from the group consisting of from 50 to 99.

Exemplary sequence identity values include 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%. Sequence identity can be determined using, for example, the GAP or BLAST

5 algorithms.

As those of skill will appreciate, the present invention includes catalytically active polypeptides of the present invention (i.e., enzymes). Catalytically active polypeptides have a specific activity of at least 20%, 30%, or 40%, and preferably at least 50%, 60%, or 70%, and most preferably at least 80%, 90%, or 95% that of the native (non-

10 synthetic), endogenous polypeptide. Further, the substrate specificity (k_{cat}/K_m) is optionally substantially similar to the native (non-synthetic), endogenous polypeptide.

Typically, the K_m will be at least 30%, 40%, or 50%, that of the native (non-synthetic), endogenous polypeptide; and more preferably at least 60%, 70%, 80%, or 90%.

15 Methods of assaying and quantifying measures of enzymatic activity and substrate specificity (k_{cat}/K_m), are well known to those of skill in the art.

Generally, the proteins of the present invention will, when presented as an immunogen, elicit production of an antibody specifically reactive to a polypeptide of the present invention. Further, the proteins of the present invention will not bind to antisera raised against a polypeptide of the present invention which has been fully immunosorbed

20 with the same polypeptide. Immunoassays for determining binding are well known to those of skill in the art. A preferred immunoassay is a competitive immunoassay as discussed, *infra*. Thus, the proteins of the present invention can be employed as immunogens for constructing antibodies immunoreactive to a protein of the present invention for such exemplary utilities as immunoassays or protein purification

25 techniques.

Expression of Proteins in Host Cells

Using the nucleic acids of the present invention, one may express a protein of the present invention in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

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It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

5 In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or regulatable), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain
10 transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation
15 terminator. One of skill would recognize that modifications can be made to a protein of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an
20 initiation site, or additional amino acids (*e.g.*, poly His) placed on either terminus to create conveniently located purification sequences. Restriction sites or termination codons can also be introduced.

A. Expression in Prokaryotes

25 Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as
30 the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., *Nature* 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel et al., *Nucleic Acids Res.* 8:4057 (1980)) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake et al., *Nature* 292:128 (1981)). The inclusion of selection

markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell.

Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, *Gene* 22: 229-235 (1983); Mosbach, *et al.*, *Nature* 302: 543-545 (1983)).

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B. Expression in Eukaryotes

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems.

15 In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. Sherman, F., *et al.*, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1982) is a well recognized work describing the various methods available to produce the protein in yeast. Two

20 widely utilized yeast for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of 25 replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

30 The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form

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of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.*, *Immunol. Rev.* 89: 49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection.

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See, Schneider, *J. Embryol. Exp. Morphol.* 27: 353-365 (1987)).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, *J. Virol.* 45: 773-781 (1983)). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector in *DNA Cloning Vol. II a Practical Approach*, D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238 (1985).

Transfection/Transformation of Cells

The method of transformation/transfection is not critical to the instant invention; various methods of transformation or transfection are currently available. As newer methods are available to transform crops or other host cells they may be directly applied. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription and/or translation of the

sequence to effect phenotypic changes in the organism. Thus, any method which provides for effective transformation/transfection may be employed.

A. Plant Transformation

A DNA sequence coding for the desired polypeptide of the present invention, for example a cDNA or a genomic sequence encoding a full length protein, will be used to construct a recombinant expression cassette which can be introduced into the desired plant.

Isolated nucleic acids of the present invention can be introduced into plants according to techniques known in the art. Generally, recombinant expression cassettes as described above and suitable for transformation of plant cells are prepared. The isolated nucleic acids of the present invention can then be used for transformation. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols may vary depending on the type of plant cell, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium* mediated transformation (see for example, Zhao *et al.* U.S. Patent 5,981,840; Hinchee *et al.* (1988) *Biotechnology* 6:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.* U.S. Patent 4,945,050; Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize) Fromm *et al.* (1990) *Biotechnology* 8:833-

839 (maize); Hooykaas-Van Slogteren & Hooykaas (1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) In *The Experimental Manipulation of Ovule Tissues* ed. G.P. Chapman *et al.* pp. 197-209. Longman, NY (pollen); Kaepller *et al.* (1990) *Plant Cell Reports* 9:415-418; and Kaepller *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); LI *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

10 The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports*, 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure 15 that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

B. Transfection of Prokaryotes, Lower Eukaryotes, and Animal Cells

20 Animal and lower eukaryotic (e.g., yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation, biolistics, and micro-injection of the DNA directly into the cells. The transfected cells 25 are cultured by means well known in the art. Kuchler, R.J., *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc. (1977).

Synthesis of Proteins

30 The proteins of the present invention can be constructed using non-cellular synthetic methods. Solid phase synthesis of proteins of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield,

Solid-Phase Peptide Synthesis, pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*; Merrifield, *et al.*, *J. Am. Chem. Soc.* 85: 2149-2156 (1963), and Stewart *et al.*, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chem. Co., Rockford, Ill. (1984). Proteins of greater length may be synthesized

5 by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide)) is known to those of skill.

Purification of Proteins

10 The proteins of the present invention may be purified by standard techniques well known to those of skill in the art. Recombinantly produced proteins of the present invention can be directly expressed or expressed as a fusion protein. The recombinant protein is purified by a combination of cell lysis (e.g., sonication, French press) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein 15 with an appropriate proteolytic enzyme releases the desired recombinant protein.

The proteins of this invention, recombinant or synthetic, may be purified to substantial purity by standard techniques well known in the art, including detergent solubilization, selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. *See*, for instance, R. Scopes, 20 *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982); Deutscher, *Guide to Protein Purification*, Academic Press (1990). For example, antibodies may be raised to the proteins as described herein. Purification from *E. coli* can be achieved following procedures described in U.S. Patent No. 4,511,503. The protein may then be isolated from cells expressing the protein and further purified by 25 standard protein chemistry techniques as described herein. Detection of the expressed protein is achieved by methods known in the art and include, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation.

Transgenic Plant Regeneration

30 Plants cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant. Plant regeneration

from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, Macmillan Publishing Company, New York, pp. 124-176 (1983); and Binding, *Regeneration of Plants, Plant Protoplasts*, CRC Press, Boca Raton, pp. 21-73 (1985).

5 The regeneration of plants containing the foreign gene introduced by *Agrobacterium* from leaf explants can be achieved as described by Horsch *et al.*, *Science*, 227:1229-1231 (1985). In this procedure, transformants are grown in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant species being transformed as described by Fraley *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 80:4803 (1983). This procedure typically produces shoots within two to four weeks and these transformant shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Transgenic plants of the present invention may be fertile or sterile.

10 Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38: 467-486 (1987). The regeneration of plants from either single plant protoplasts or various explants is well known in the art. See, for example, *Methods for Plant Molecular Biology*, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, Calif. (1988). This regeneration and growth process includes the steps of 15 selection of transformant cells and shoots, rooting the transformant shoots and growth of the plantlets in soil. For maize cell culture and regeneration see generally, *The Maize Handbook*, Freeling and Walbot, Eds., Springer, New York (1994); *Corn and Corn Improvement*, 3rd edition, Sprague and Dudley Eds., American Society of Agronomy, Madison, Wisconsin (1988).

20 One of skill will recognize that after the recombinant expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

25 In vegetatively propagated crops, mature transgenic plants can be propagated by 30 the taking of cuttings or by tissue culture techniques to produce multiple identical plants. Selection of desirable transgenics is made and new varieties are obtained and propagated vegetatively for commercial use. In seed propagated crops, mature transgenic plants can be self crossed to produce a homozygous inbred plant. The inbred plant produces

seed containing the newly introduced heterologous nucleic acid. These seeds can be grown to produce plants that would produce the selected phenotype.

Parts obtained from the regenerated plant, such as flowers, seeds, leaves, branches, fruit, and the like are included in the invention, provided that these parts comprise cells comprising the isolated nucleic acid of the present invention. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

Transgenic plants expressing the selectable marker can be screened for transmission of the nucleic acid of the present invention by, for example, standard immunoblot and DNA detection techniques. Transgenic lines are also typically evaluated on levels of expression of the heterologous nucleic acid. Expression at the RNA level can be determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis can be employed and include PCR amplification assays using oligonucleotide primers designed to amplify only the heterologous RNA templates and solution hybridization assays using heterologous nucleic acid-specific probes. The RNA-positive plants can then analyzed for protein expression by Western immunoblot analysis using the specifically reactive antibodies of the present invention. In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using heterologous nucleic acid specific polynucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue. Generally, a number of transgenic lines are usually screened for the incorporated nucleic acid to identify and select plants with the most appropriate expression profiles.

A preferred embodiment is a transgenic plant that is homozygous for the added heterologous nucleic acid; i.e., a transgenic plant that contains two added nucleic acid sequences, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) a heterozygous transgenic plant that contains a single added heterologous nucleic acid, germinating some of the seed produced and analyzing the resulting plants produced for altered expression of a polynucleotide of the present invention relative to a control plant (i.e., native, non-transgenic). Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

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Modulating Polypeptide Levels and/or Composition

The present invention further provides a method for modulating (i.e., increasing or decreasing) the concentration or ratio of the polypeptides of the present invention in a plant or part thereof. Modulation can be effected by increasing or decreasing the 5 concentration and/or the ratio of the polypeptides of the present invention in a plant. The method comprises introducing into a plant cell with a recombinant expression cassette comprising a polynucleotide of the present invention as described above to obtain a transformed plant cell, culturing the transformed plant cell under plant cell growing conditions, and inducing or repressing expression of a polynucleotide of the present 10 invention in the plant for a time sufficient to modulate concentration and/or the ratios of the polypeptides in the plant or plant part.

In some embodiments, the concentration and/or ratios of polypeptides of the present invention in a plant may be modulated by altering, *in vivo* or *in vitro*, the promoter of a gene to up- or down-regulate gene expression. In some embodiments, the coding regions of native genes of the present invention can be altered via substitution, addition, insertion, or deletion to decrease activity of the encoded enzyme. See, e.g., Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868. And in some embodiments, an isolated nucleic acid (e.g., a vector) comprising a promoter sequence is transfected into a plant cell. Subsequently, a plant cell comprising the promoter operably linked to a polynucleotide of the present invention is selected for by means known to those of skill in the art such as, but not limited to, Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the gene and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or ratios of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art and discussed briefly, *supra*.

In general, concentration or the ratios of the polypeptides is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned recombinant expression cassette. Modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development. Modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of

the present invention in, for example, sense or antisense orientation as discussed in greater detail, *supra*. Induction of expression of a polynucleotide of the present invention can also be controlled by exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds which activate expression from these promoters are well known in the art. In preferred embodiments, the polypeptides of the present invention are modulated in monocots, particularly maize.

Molecular Markers

10 The present invention provides a method of genotyping a plant comprising a Rad50 polynucleotide of the present invention. Preferably, the plant is a monocot, such as maize or sorghum. Genotyping provides a means of distinguishing homologs of a chromosome pair and can be used to differentiate segregants in a plant population.

15 Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, The DNA Revolution by Andrew H. Paterson 1996 (Chapter 2) 20 in: *Genome Mapping in Plants* (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp.7-21.

25 The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). RFLPs are the product of allelic differences between DNA restriction fragments resulting from nucleotide sequence variability. As is well known to those of skill in the art, RFLPs are typically detected by extraction of genomic DNA and digestion with a restriction enzyme. Generally, the resulting fragments are separated according to size and hybridized with a probe; single copy probes are preferred. Restriction fragments from homologous chromosomes are 30 revealed. Differences in fragment size among alleles represent an RFLP. Thus, the present invention further provides a means to follow segregation of Rad50 genes of the present invention as well as chromosomal sequences genetically linked to Rad50 genes using such techniques as RFLP analysis. Linked chromosomal sequences are within 50

centiMorgans (cM), often within 40 or 30 cM, preferably within 20 or 10 cM, more preferably within 5, 3, 2, or 1 cM of a Rad50 gene of the present invention.

In the present invention, the nucleic acid probes employed for molecular marker mapping of plant nuclear genomes selectively hybridize, under selective hybridization conditions, to a gene encoding a Rad50 polynucleotide. In preferred embodiments, the probes are selected from polynucleotides of the present invention. Typically, these probes are cDNA probes or restriction-enzyme treated (e.g., *Pst I*) genomic clones. In the present invention probes can be made from the polynucleotide of SEQ ID NO: 1. The length of the probes is discussed in greater detail, *supra*, but are typically at least 15 bases in length, more preferably at least 20, 25, 30, 35, 40, or 50 bases in length. Generally, however, the probes are less than about 1 kilobase in length. Preferably, the probes are single copy probes that hybridize to a unique locus in a haploid chromosome complement. Some exemplary restriction enzymes employed in RFLP mapping are *EcoRI*, *EcoRV*, and *SstI*. As used herein the term “restriction enzyme” includes reference to a composition that recognizes and, alone or in conjunction with another composition, cleaves at a specific nucleotide sequence.

The method of detecting an RFLP comprises the steps of (a) digesting genomic DNA of a plant with a restriction enzyme; (b) hybridizing a nucleic acid probe, under selective hybridization conditions, to a sequence of a polynucleotide of the present of 20 said genomic DNA; (c) detecting therefrom a RFLP.

Other methods of differentiating polymorphic (allelic) variants of polynucleotides of the present invention can be had by utilizing molecular marker techniques well known to those of skill in the art including such techniques as: 1) single stranded conformation analysis (SSCA); 2) denaturing gradient gel electrophoresis (DGGE); 3) RNase protection assays; 4) allele-specific oligonucleotides (ASOs); 5) the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein; and 6) allele-specific PCR. Other approaches based on the detection of mismatches between the two complementary DNA strands include clamped denaturing gel electrophoresis (CDGE); heteroduplex analysis (HA); and chemical mismatch cleavage (CMC). Thus, the present invention further provides a method of genotyping comprising the steps of contacting, under stringent hybridization conditions, a sample suspected of comprising a Rad50 polynucleotide with a nucleic acid probe. Generally, the sample is a plant sample; preferably, a sample suspected of comprising a maize polynucleotide of the present

invention (e.g., gene, mRNA). The nucleic acid probe selectively hybridizes, under stringent conditions, to a subsequence of a Rad50 polynucleotide comprising a polymorphic marker. Selective hybridization of the nucleic acid probe to the polymorphic marker nucleic acid sequence yields a hybridization complex. Detection of 5 the hybridization complex indicates the presence of that polymorphic marker in the sample. In preferred embodiments, the nucleic acid probe comprises a polynucleotide of the present invention.

10 UTRs and Codon Preference

In general, translational efficiency has been found to be regulated by specific sequence elements in the 5' non-coding or untranslated region (5' UTR) of the RNA. Positive sequence motifs include translational initiation consensus sequences (Kozak, *Nucleic Acids Res.* 15:8125 (1987)) and the 7-methylguanosine cap structure (Drummond 15 *et al.*, *Nucleic Acids Res.* 13:7375 (1985)). Negative elements include stable intramolecular 5' UTR stem-loop structures (Muesing *et al.*, *Cell* 48:691 (1987)) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5' UTR (Kozak, *supra*, Rao *et al.*, *Mol. and Cell. Biol.* 8:284 (1988)). Accordingly, the 20 present invention provides 5' and/or 3' UTR regions for modulation of translation of heterologous coding sequences.

Further, the polypeptide-encoding segments of the polynucleotides of the present invention can be modified to alter codon usage. Altered codon usage can be employed to alter translational efficiency and/or to optimize the coding sequence for expression in a desired host such as to optimize the codon usage in a heterologous sequence for 25 expression in maize. Codon usage in the coding regions of the polynucleotides of the present invention can be analyzed statistically using commercially available software packages such as "Codon Preference" available from the University of Wisconsin Genetics Computer Group (see Devereaux *et al.*, *Nucleic Acids Res.* 12: 387-395 (1984)) or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.). Thus, the present 30 invention provides a codon usage frequency characteristic of the coding region of at least one of the polynucleotides of the present invention. The number of polynucleotides that can be used to determine a codon usage frequency can be any integer from 1 to the number of polynucleotides of the present invention as provided herein. Optionally, the

polynucleotides will be full-length sequences. An exemplary number of sequences for statistical analysis can be at least 1, 5, 10, 20, 50, or 100.

Sequence Shuffling

5 The present invention provides methods for sequence shuffling using polynucleotides of the present invention, and compositions resulting therefrom. Sequence shuffling is described in PCT publication No. WO 97/20078. See also, Zhang, J.- H., *et al. Proc. Natl. Acad. Sci. USA* 94:4504-4509 (1997). Generally, sequence shuffling provides a means for generating libraries of polynucleotides having a desired 10 characteristic which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides which comprise sequence regions which have substantial sequence identity and can be homologously recombined in vitro or in vivo. The population of sequence-recombined polynucleotides comprises a subpopulation of polynucleotides which possess desired or 15 advantageous characteristics and which can be selected by a suitable selection or screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a 20 gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be a decreased K_m and/or increased K_{cat} over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled 25 wild-type polynucleotide. The increase in such properties can be at least 110%, 120%, 130%, 140% or at least 150% of the wild-type value.

Generic and Consensus Sequences

30 Polynucleotides and polypeptides of the present invention further include those having: (a) a generic sequence of at least two homologous polynucleotides or polypeptides, respectively, of the present invention; and, (b) a consensus sequence of at least three homologous polynucleotides or polypeptides, respectively, of the present invention. The generic sequence of the present invention comprises each species of

polypeptide or polynucleotide embraced by the generic polypeptide or polynucleotide, sequence, respectively. The individual species encompassed by a polynucleotide having an amino acid or nucleic acid consensus sequence can be used to generate antibodies or produce nucleic acid probes or primers to screen for homologs in other species, genera, families, orders, classes, phylums, or kingdoms. For example, a polynucleotide having a consensus sequences from a gene family of *Zea mays* can be used to generate antibody or nucleic acid probes or primers to other *Gramineae* species such as wheat, rice, or sorghum. Alternatively, a polynucleotide having a consensus sequence generated from orthologous genes can be used to identify or isolate orthologs of other taxa. Typically, a polynucleotide having a consensus sequence will be at least 9, 10, 15, 20, 25, 30, or 40 amino acids in length, or 20, 30, 40, 50, 100, or 150 nucleotides in length. As those of skill in the art are aware, a conservative amino acid substitution can be used for amino acids which differ amongst aligned sequence but are from the same conservative substitution group as discussed above. Optionally, no more than 1 or 2 conservative amino acids are substituted for each 10 amino acid length of consensus sequence.

Similar sequences used for generation of a consensus or generic sequence include any number and combination of allelic variants of the same gene, orthologous, or paralogous sequences as provided herein. Optionally, similar sequences used in generating a consensus or generic sequence are identified using the BLAST algorithm's smallest sum probability (P(N)). Various suppliers of sequence-analysis software are listed in chapter 7 of *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (Supplement 30). A polynucleotide sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, or 0.001, and most preferably less than about 0.0001, or 0.00001. Similar polynucleotides can be aligned and a consensus or generic sequence generated using multiple sequence alignment software available from a number of commercial suppliers such as the Genetics Computer Group's (Madison, WI) PILEUP software, Vector NTI's (North Bethesda, MD) ALIGNX, or Genecode's (Ann Arbor, MI) SEQUENCHER. Conveniently, default parameters of such software can be used to generate consensus or generic sequences.

Assays for Compounds that Modulate Enzymatic Activity or Expression

The present invention also provides means for identifying compounds that bind to (e.g., substrates), and/or increase or decrease (i.e., modulate) the enzymatic activity of, catalytically active polypeptides of the present invention. The method comprises

5 contacting a polypeptide of the present invention with a compound whose ability to bind to or modulate enzyme activity is to be determined. The polypeptide employed will have at least 20%, preferably at least 30% or 40%, more preferably at least 50% or 60%, and most preferably at least 70% or 80% of the specific activity of the native, full-length polypeptide of the present invention (e.g., enzyme). Generally, the polypeptide will be

10 present in a range sufficient to determine the effect of the compound, typically about 1 nM to 10 μ M. Likewise, the compound will be present in a concentration of from about 1 nM to 10 μ M. Those of skill will understand that such factors as enzyme concentration, ligand concentrations (i.e., substrates, products, inhibitors, activators), pH, ionic strength, and temperature will be controlled so as to obtain useful kinetic data

15 and determine the presence of absence of a compound that binds or modulates polypeptide activity. Methods of measuring enzyme kinetics is well known in the art. See, e.g., Segel, *Biochemical Calculations*, 2nd ed., John Wiley and Sons, New York (1976).

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Example 1

25 This example describes the construction of the cDNA libraries.

Total RNA Isolation

The RNA for SEQ ID NO: 1 was isolated from premeiotic ear shoot tissue from maize line A632. Total RNA was isolated from corn tissues with TRIZOL Reagent (Life 30 Technology Inc. Gaithersburg, MD) using a modification of the guanidine isothiocyanate/acid-phenol procedure described by Chomczynski and Sacchi (Chomczynski, P., and Sacchi, N. *Anal. Biochem.* 162, 156 (1987)). In brief, plant tissue samples were pulverized in liquid nitrogen before the addition of the TRIZOL

Reagent, and then were further homogenized with a mortar and pestle. Addition of chloroform followed by centrifugation was conducted for separation of an aqueous phase and an organic phase. The total RNA was recovered by precipitation with isopropyl alcohol from the aqueous phase.

5

Poly(A)+ RNA Isolation

The selection of poly(A)+ RNA from total RNA was performed using POLYATTRACT system (Promega Corporation. Madison, WI). In brief, biotinylated oligo(dT) primers were used to hybridize to the 3' poly(A) tails on mRNA. The hybrids were captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was washed at high stringency conditions and eluted by RNase-free deionized water.

cDNA Library Construction

15 cDNA synthesis was performed and unidirectional cDNA libraries were constructed using the SUPERSCRIPt Plasmid System (Life Technology Inc. Gaithersburg, MD). The first stand of cDNA was synthesized by priming an oligo(dT) primer containing a Not I site. The reaction was catalyzed by SUPERSCRIPt Reverse Transcriptase II at 45°C. The second strand of cDNA was labeled with alpha-³²P-dCTP 20 and a portion of the reaction was analyzed by agarose gel electrophoresis to determine cDNA sizes. cDNA molecules smaller than 500 base pairs and unligated adapters were removed by SEPHACRYL-S400 chromatography. The selected cDNA molecules were ligated into pSPORT1 vector in between of *Not* I and *Sal* I sites.

25 Example 2

This example describes cDNA sequencing and library subtraction.

Sequencing Template Preparation

Individual colonies were picked and DNA was prepared either by PCR with M13 30 forward primers and M13 reverse primers, or by plasmid isolation. All the cDNA clones were sequenced using M13 reverse primers.

Q-bot Subtraction Procedure

cDNA libraries subjected to the subtraction procedure were plated out on 22 x 22 cm² agar plate at density of about 3,000 colonies per plate. The plates were incubated in a 37°C incubator for 12-24 hours. Colonies were picked into 384-well plates by a robot 5 colony picker, Q-bot (GENETIX Limited). These plates were incubated overnight at 37°C.

Once sufficient colonies were picked, they were pinned onto 22 x 22 cm² nylon membranes using Q-bot. Each membrane contained 9,216 colonies or 36,864 colonies. These membranes were placed onto agar plate with appropriate antibiotic. The plates 10 were incubated at 37°C for overnight.

After colonies were recovered on the second day, these filters were placed on filter paper pre-wetted with denaturing solution for four minutes, then were incubated on top of a boiling water bath for additional four minutes. The filters were then placed on filter paper pre-wetted with neutralizing solution for four minutes. After excess solution 15 was removed by placing the filters on dry filter papers for one minute, the colony side of the filters were place into Proteinase K solution, incubated at 37°C for 40-50 minutes. The filters were placed on dry filter papers to dry overnight. DNA was then cross-linked to nylon membrane by UV light treatment.

Colony hybridization was conducted as described by Sambrook,J., Fritsch, E.F. 20 and Maniatis, T., (in Molecular Cloning: A laboratory Manual, 2nd Edition). The following probes were used in colony hybridization:

1. First strand cDNA from the same tissue as the library was made from to remove the most redundant clones.
2. 48-192 most redundant cDNA clones from the same library based on previous 25 sequencing data.
3. 192 most redundant cDNA clones in the entire corn sequence database.
4. A Sal-A20 oligo nucleotide: TCG ACC CAC GCG TCC GAA AAA AAA AAA AAA AAA AAA AAA, listed in SEQ ID NO: 3, removes clones containing a poly A tail but no cDNA.
- 30 5. cDNA clones derived from rRNA.

The image of the autoradiography was scanned into computer and the signal intensity and cold colony addresses of each colony was analyzed. Re-arranging of cold-colonies from 384 well plates to 96 well plates was conducted using Q-bot.

5 **Example 3**

This example describes identification of the gene from a computer homology search.

Gene identities were determined by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1990) *J. Mol. Biol.* 215:403-410; see also

10 www.ncbi.nlm.nih.gov/BLAST/) searches under default parameters for similarity to sequences contained in the BLAST “nr” database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences were analyzed
 15 for similarity to all publicly available DNA sequences contained in the “nr” database using the BLASTN algorithm. The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the “nr” database using the BLASTX algorithm (Gish, W. and States, D. J. *Nature Genetics* 3:266-272 (1993)) provided by the NCBI. In some cases, the sequencing data
 20 from two or more clones containing overlapping segments of DNA were used to construct contiguous DNA sequences.

Example 4

This example displays structural motifs of the maize Rad50 protein sequence.

25 The **highlighted** areas indicate nucleotide binding sites. The N-terminal binding site, the Walker-A motif, is known to bind ATP. The putative nuclear localization signals are identified by *italics* and the **bolding** indicates the leucine zipper motifs.

Structural motifs of Maize Rad50 protein sequence (SEQ ID NO: 2)

30

1 MSTVDKMLIK GIRSFDPDNK NVITFFKPLT LIVGPNGAGK TTIECLKLS

51 CTGELPPNSR SGHTFVHDPK VAGETETKGQ IKLRFKTAAG KDVVCIRSFQ

DRAFT: 05/20/2016 10:22:56 AM

101 LTQKASKMEF KAIIESVLQTI NPHTGEKVCL SYRCADMRE IPALMGVSKA
 151 VLEENVIFVHQ DESNWPLQDP STLKKKFDDI FSATTRYTKAL EVIKKLHKDQ
 5
 201 MQEIKTFRLK LENLQTVKDQ AHKLRENIAQ DQEKSASKS QMEQLKEKIC
 251 GTEREILQME TSLDELRRRLQ GQIDIKATER STLLTQQHEK LAALSEENED
 10
 301 TDEELMEWQT KFEERIALLE TKISKLVRDM DDEASYSSVL SKQNSELTHE
 351 IGKLQAEADA HLTMKHERDS DIKNICKHN LGPVPEHPFT NDVAMNLTNR
 15
 401 IKARLSSLEN DLLDKKSNE DQLDVLWKHY LKINARYSEV DGQIQSKIES
 451 MSGILRRRKD KEKERDAAEV ELSKFNLNSRI DERERHMQIE VERKTLALGE
 501 RDYDSIISQK RTEVYSLEQK IKVLLREKDI INRNADERVK LGLKKDALES
 20
 551 SKDKLNEIVN EHDKIKKVL RGRNPFEKDM KKEINQAFWP VDKEYNELRS
 601 KSQEAEQELK FTQSKVTDAR EQLTKLRRDM DAKRRFLDSK LQSILQISAN
 651 VDMFPKVQLQD AMNKRDEQKR LENFANGMRE MLAPFEHLAR KNHVCPCER
 25
 701 AFTPDEEDEF VKKQRMQNSS TAERSKALAM ESSNAEALFQ QLDKLRTIYD
 751 AYVKLVEETI PLAENLNQH LADESQKAQA FDDLLGVLAH VQMDRDAVEA
 30
 801 LLQPTDTIDR HVHEIQQLVK EVEDLEYALD SSGRGVKSLE EIQLELNFLQ
 851 RTRDTLIVEV DDLRDQHRML NEDMSSAQVR WHNAREEKVK ASSILERFQK
 901 SEEELVLLAE EKEQLIVEKK LLEESLDPLS KEKESLLQEY NALKQKLDEE
 35
 951 YHQLAERKRE FQQELDALGR LNMKIKGYLD SKKNEKLKEL QGRHVLCHSQ
 1001 LQSCMAKQQR ISAELNKSKE LLQGQGQLKR NIDDNLKYRK TKADVEQLTR

1051 DIESLEERLL SIGSLSATEA DLKRHSQEKE RLNSEFNRWQ GTLSVYQSNI

1101 SKHKQELKLS QYKDIKRYT NQFLQLKTTE MANKDLDYY TALDKALMRF

5 1151 HSMKMEINK IIKELWQQTY RGQDIDYISI NSDSEGAGTR SYSYRVVMQT

1201 GDAELEMGRGR CSAGQKVLAS LIIRLALAET FCLNCGILAL DEPTTNLDGP

1251 NAESLAAALL RIMEARKGQE NFQLIVITHD ERF AHLIGQR QLAEKYYRVS

10

1301 KDENQHSIIE SQEIFD*

The above examples are provided to illustrate the invention but not to limit its
15 scope. Other variants of the invention will be readily apparent to one of ordinary skill in
the art and are encompassed by the appended claims. All publications, patents, patent
applications, and computer programs cited herein are hereby incorporated by reference.

One or more of the figures is a drawing.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
 - (a) a polynucleotide having at least 80% sequence identity to the polynucleotide of SEQ ID NO: 1, wherein the % sequence identity is based on the entire coding region for each reference sequence and is calculated by the GAP algorithm under default parameters;
 - (b) a polynucleotide encoding the polypeptide of SEQ ID NO: 2;
 - (c) a polynucleotide amplified from a *Zea mays* nucleic acid library using primers which selectively hybridize, under stringent hybridization conditions, to loci within the polynucleotide of SEQ ID NO: 1;
 - (d) a polynucleotide which selectively hybridizes, under stringent hybridization conditions and a wash in 0.1X SSC at 60°C, to the polynucleotide of SEQ ID NO: 1;
 - (e) the polynucleotide of SEQ ID NO: 1;
 - (f) a polynucleotide which is complementary to a polynucleotide of (a), (b), (c), (d), or (e); and
 - (g) a polynucleotide comprising at least 30 contiguous nucleotides from a polynucleotide of (a), (b), (c), (d), (e), or (f).
2. A recombinant expression cassette, comprising a member of claim 1 operably linked, in sense or anti-sense orientation, to a promoter.
3. A host cell comprising the recombinant expression cassette of claim 2.
4. A transgenic plant comprising a recombinant expression cassette of claim 2.
5. The transgenic plant of claim 4, wherein said plant is a monocot.
6. The transgenic plant of claim 4, wherein said plant is a dicot.

7. The transgenic plant of claim 4, wherein said plant is selected from the group consisting of: maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, and millet.

8. A transgenic seed from the transgenic plant of claim 4.

9. A method of modulating the level of Rad50 in a plant, comprising:

- (a) introducing into a plant cell a recombinant expression cassette comprising a Rad50 polynucleotide of claim 1 operably linked to a promoter;
- (b) culturing the plant cell under plant cell growing conditions;
- (c) regenerating a whole plant which possesses the transformed genotype; and
- (d) inducing expression of said polynucleotide for a time sufficient to modulate the level of Rad50 in said plant.

10. The method of claim 9, wherein the plant is maize.

11. An isolated protein comprising a member selected from the group consisting of:

- (a) a polypeptide of at least 20 contiguous amino acids from the polypeptide of SEQ ID NO: 2;
- (b) the polypeptide of SEQ ID NO: 2;
- (c) a polypeptide having at least 80% sequence identity to, and having at least one linear epitope in common with, the polypeptide of SEQ ID NO: 2, wherein said sequence identity is determined using the GAP program under default parameters; and
- (d) at least one polypeptide encoded by a member of claim 1.

ABSTRACT OF THE DISCLOSURE

The invention provides isolated maize Rad50 nucleic acids and their encoded proteins. The present invention provides methods and compositions relating to altering Rad50 levels in plants. The invention further provides recombinant expression cassettes, host cells, transgenic plants, and antibody compositions.

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DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Maize Rad50 Orthologue and Uses Thereof

the specification of which (check one)



is attached hereto
was filed on _____ as Application Serial No. _____ and was
amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign or provisional application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	MONTH/DAY/YEAR FILED	PRIORITY CLAIMED

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of

Federal Regulations §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED
60/132,575	May 5, 1999	Pending

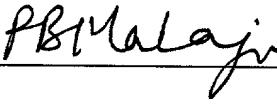
I hereby appoint as my attorneys and/agents, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Debra L. Blair	Registration No. 39,112
Donna Bobrowicz	Registration No. 32,196
Jean M. Bromert	Registration No. 42,362
Catherine D. Brooke	Registration No. 44,041
Karen Moon Bruce	Registration No. 42,366
Steven J. Callistein	Registration No. 43,525
Herbert H. Jervis	Registration No. 31,171
Marianne H. Michel	Registration No. 35,286
David B. Ran	Registration No. 38,589
Patricia A. Sweeney	Registration No. 32,733
Michael E. Yates	Registration No. 36,063

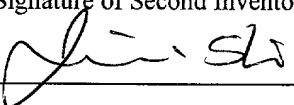
Send Correspondence to: Michael E. Yates
Patent Counsel
7100 N.W. 62nd Avenue
P.O. Box 1000
Johnston, Iowa 50131-1000

Direct telephone calls to: Michael E. Yates
(515) 248-4835

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor Pramod B. Mahajan	Signature of First or Sole Inventor 	Date 3-28-2003
Residence Address 8029 Brookview Drive, Urbandale, Iowa	Country or Citizenship United States of America	
Post Office Address		

Signatures should conform to names as typewritten. Additional inventors on attached page 3.

Full Name of Second Inventor Jinrui Shi	Signature of Second Inventor 	Date 3-28-00
Residence Address 6022 Greendale Circle, Apt. 205, Johnston, Iowa	Country or Citizenship China	
Post Office Address		

00000000000000000000000000000000

SEQUENCE LISTING

<110> Mahajan, Pramod B.
Shi, Jinrui

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35					40					45				50		

tgc	acc	gg	gag	ctg	ccc	aac	tcc	cg	tct	ggc	cac	acc	ttc	gtc	489	
Cys	Thr	Gly	Glu	Leu	Pro	Pro	Asn	Ser	Arg	Ser	Gly	His	Thr	Phe	Val	
55					60					65						

cac	gac	ccc	aag	gta	gct	ggc	gag	ac	gaa	aca	aaa	gga	caa	att	aag	537
His	Asp	Pro	Lys	Val	Ala	Gly	Glu	Thr	Glu	Thr	Lys	Gly	Gln	Ile	Lys	
70					75					80						

ttg	cgg	ttt	aag	act	gca	gca	gga	aag	gat	gtg	gtg	tgc	atc	cg	tcc	585
Leu	Arg	Phe	Lys	Thr	Ala	Ala	Gly	Lys	Asp	Val	Val	Cys	Ile	Arg	Ser	
85					90					95						

ttc	cag	ctt	acc	caa	aag	gca	tca	aag	atg	gag	ttt	aag	gca	att	gaa	633
Phe	Gln	Leu	Thr	Gln	Ala	Ser	Lys	Met	Glu	Phe	Lys	Ala	Ile	Glu		

100	105	110	
agc gtc ctc cag act ata aat cca cac aca ggg gag aaa gtc tgc ctc Ser Val Leu Gln Thr Ile Asn Pro His Thr Gly Glu Lys Val Cys Leu	115	120	681
125		125	130
agc tac aga tgt gct gac atg gat aga gag att cct gcc tta atg ggt Ser Tyr Arg Cys Ala Asp Met Asp Arg Glu Ile Pro Ala Leu Met Gly	135	140	729
140		145	
gtt tcg aag gcc gta ctg gag aat gtt ata ttt gtt cac caa gat gaa Val Ser Lys Ala Val Leu Glu Asn Val Ile Phe Val His Gln Asp Glu	150	155	777
155		160	
tcc aat tgg cca ttg cag gac ccg tca aca ctt aag aag aag ttc gat Ser Asn Trp Pro Leu Gln Asp Pro Ser Thr Leu Lys Lys Lys Phe Asp	165	170	825
170		175	
gac atc ttc tct gcc aca cgc tat acg aaa gct ctt gaa gtc ata aag Asp Ile Phe Ser Ala Thr Arg Tyr Thr Lys Ala Leu Glu Val Ile Lys	180	185	873
185		190	
aaa ctt cac aag gat caa atg caa gag atc aag act ttt agg tta aag Lys Leu His Lys Asp Gln Met Gln Glu Ile Lys Thr Phe Arg Leu Lys	195	200	921
200		205	210
ctg gag aac ctt cag act gta aaa gac caa gca cat aag ctg cgt gaa Leu Glu Asn Leu Gln Thr Val Lys Asp Gln Ala His Lys Leu Arg Glu	215	220	969
220		225	
aat att gct caa gat caa gaa aag tca gat gcc tca aaa tct cag atg Asn Ile Ala Gln Asp Gln Glu Lys Ser Asp Ala Ser Lys Ser Gln Met	230	235	1017
235		240	
gag caa ctg aag gaa aag atc tgt ggt acc gag aga gaa atc ctg caa Glu Gln Leu Lys Glu Lys Ile Cys Gly Thr Glu Arg Glu Ile Leu Gln	245	250	1065
250		255	
atg gaa aca agt ttg gat gaa ctg aga aga ctt cag gga caa att gac Met Glu Thr Ser Leu Asp Glu Leu Arg Arg Leu Gln Gly Gln Ile Asp	260	265	1113
265		270	
atc aag gca aca gag aga agt aca tta ctt acg cag cag cat gaa aag Ile Lys Ala Thr Glu Arg Ser Thr Leu Leu Thr Gln Gln His Glu Lys	275	280	1161
280		285	290
ctt gct gca ctt tct gag gaa aat gaa gat acc gat gag gaa cta atg Leu Ala Ala Leu Ser Glu Glu Asn Glu Asp Thr Asp Glu Glu Leu Met	295	300	1209
300		305	
gaa tgg caa aca aaa ttt gaa gaa agg att gcg tta cta gaa aca aaa Glu Trp Gln Thr Lys Phe Glu Glu Arg Ile Ala Leu Leu Glu Thr Lys	310	315	1257
315		320	
atc agt aaa ctt gta aga gat atg gat gat gaa gca tct tat agc tcc Ile Ser Lys Leu Val Arg Asp Met Asp Asp Glu Ala Ser Tyr Ser Ser	325	330	1305
330		335	
gtt ctg tcc aaa caa aat tct gaa tta aca cat gaa att gga aag ctc Val Leu Ser Lys Gln Asn Ser Glu Leu Thr His Glu Ile Gly Lys Leu	340	345	1353
345		350	

DRAFT 2009-09-10

cag gca gaa gct gat gct cac ctg act atg aag cat gaa cga gac tca Gln Ala Glu Ala Asp Ala His Leu Thr Met Lys His Glu Arg Asp Ser 355	360	365	370	1401
gac ata aaa aat ata tgc act aaa cat aat ctt ggg ccg gtt cct gaa Asp Ile Lys Asn Ile Cys Thr Lys His Asn Leu Gly Pro Val Pro Glu 375	380	385		1449
cat ccc ttt acg aat gat gtt gct atg aac ctt aca aac agg att aaa His Pro Phe Thr Asn Asp Val Ala Met Asn Leu Thr Asn Arg Ile Lys 390	395	400		1497
gcg aga cta tca agt ctt gag aat gat ttg ctg gat aag aag aaa tcc Ala Arg Leu Ser Ser Leu Glu Asn Asp Leu Leu Asp Lys Lys Ser 405	410	415		1545
aat gaa gat cag tta gat gtt ttg tgg aaa cac tat ctt aaa ata aat Asn Glu Asp Gln Leu Asp Val Leu Trp Lys His Tyr Leu Lys Ile Asn 420	425	430		1593
gct cgc tac tcc gaa gtt gat ggt cag ata caa tct aag att gaa tcc Ala Arg Tyr Ser Glu Val Asp Gly Gln Ile Gln Ser Lys Ile Glu Ser 435	440	445	450	1641
atg tca ggc att tta aga cgg aga aaa gat aaa gag aaa gaa cgc gat Met Ser Gly Ile Leu Arg Arg Lys Asp Lys Glu Lys Glu Arg Asp 455	460	465		1689
gct gca gaa gtg gag ctt tca aaa ttt aat cta tcc cgt atc gat gag Ala Ala Glu Val Glu Leu Ser Lys Phe Asn Leu Ser Arg Ile Asp Glu 470	475	480		1737
agg gag aga cat atg caa att gaa gtc gag agg aag aca ctt gcg ctt Arg Glu Arg His Met Gln Ile Glu Val Glu Arg Lys Thr Leu Ala Leu 485	490	495		1785
gga gaa aga gac tat gat tca att ata agt cag aaa cga aca gaa gta Gly Glu Arg Asp Tyr Asp Ser Ile Ile Ser Gln Lys Arg Thr Glu Val 500	505	510		1833
tat agt ttg gaa cag aaa ata aaa gtg ctt ctg cgg gag aaa gat ata Tyr Ser Leu Glu Gln Lys Ile Lys Val Leu Leu Arg Glu Lys Asp Ile 515	520	525	530	1881
ata aat aga aat gct gat gaa aga gta aaa ctg ggt ttg aag aag gat Ile Asn Arg Asn Ala Asp Glu Arg Val Lys Leu Gly Leu Lys Lys Asp 535	540	545		1929
gca ttg gaa agc agc aag gac aag ctc aat gag ata gtt aat gag cat Ala Leu Glu Ser Ser Lys Asp Lys Leu Asn Glu Ile Val Asn Glu His 550	555	560		1977
aag gat aaa atc aaa aag gta ctt agg ggg agg aat cct ttt gag aag Lys Asp Lys Ile Lys Lys Val Leu Arg Gly Arg Asn Pro Phe Glu Lys 565	570	575		2025
gat atg aag aag gag atc aat caa gcc ttt tgg cct gtg gac aag gaa Asp Met Lys Lys Glu Ile Asn Gln Ala Phe Trp Pro Val Asp Lys Glu 580	585	590		2073
tac aat gag tta aga tca aaa tcc cag gaa gca gag caa gag ctt aaa Tyr Asn Glu Leu Arg Ser Lys Ser Gln Glu Ala Glu Gln Glu Leu Lys				2121

595	600	605	610	
ttt act cag agc aaa gta act gat gct aga gaa caa ttg aca aaa ctt Phe Thr Gln Ser Lys Val Thr Asp Ala Arg Glu Gln Leu Thr Lys Leu 615 620 625				2169
cga aga gat atg gat gca aaa aga aga ttc ctg gac tcg aaa ctt caa Arg Arg Asp Met Asp Ala Lys Arg Arg Phe Leu Asp Ser Lys Leu Gln 630 635 640				2217
tct att tta cag ata tct gct aat gtt gac atg ttt ccc aaa gtt cta Ser Ile Leu Gln Ile Ser Ala Asn Val Asp Met Phe Pro Lys Val Leu 645 650 655				2265
caa gac gcc atg aac aaa aga gat gaa cag aaa aga tta gag aat ttc Gln Asp Ala Met Asn Lys Arg Asp Glu Gln Lys Arg Leu Glu Asn Phe 660 665 670				2313
gca aat gga atg cgg gaa atg ctt gca cct ttt gaa cat ttg gct cgg Ala Asn Gly Met Arg Glu Met Leu Ala Pro Phe Glu His Leu Ala Arg 675 680 685 690				2361
aag aat cat gta tgc cca tgc tgt gaa cgt gct ttc aca cct gat gag Lys Asn His Val Cys Pro Cys Cys Glu Arg Ala Phe Thr Pro Asp Glu 695 700 705				2409
gag gat gag ttc gtg aag aaa caa agg atg caa aac tca agt act gca Glu Asp Glu Phe Val Lys Lys Gln Arg Met Gln Asn Ser Ser Thr Ala 710 715 720				2457
gag aga tct aaa gct ctg gca atg gaa tca tca aat gct gaa gct ctt Glu Arg Ser Lys Ala Leu Ala Met Glu Ser Ser Asn Ala Glu Ala Leu 725 730 735				2505
ttt cag caa ttg gat aaa ctt cgg act atc tat gat gct tat gtg aag Phe Gln Gln Leu Asp Lys Leu Arg Thr Ile Tyr Asp Ala Tyr Val Lys 740 745 750				2553
ctg gta gaa gaa acc ata cct cta gca gag aaa aac ttg aat caa cat Leu Val Glu Glu Thr Ile Pro Leu Ala Glu Lys Asn Leu Asn Gln His 755 760 765 770				2601
ttg gcg gat gaa agt cag aag gcg cag gca ttt gat gat ctt ttg ggt Leu Ala Asp Glu Ser Gln Lys Ala Gln Ala Phe Asp Asp Leu Leu Gly 775 780 785				2649
gtt ctt gcc cat gtt caa atg gac agg gat gca gtg gaa gcc tta tta Val Leu Ala His Val Gln Met Asp Arg Asp Ala Val Glu Ala Leu Leu 790 795 800				2697
caa ccc act gat act att gac agg cat gta cat gaa att caa cag cta Gln Pro Thr Asp Thr Ile Asp Arg His Val His Glu Ile Gln Gln Leu 805 810 815				2745
gtc aaa gaa gta gaa gat ctt gaa tat gca ctt gat tct agt ggc cga Val Lys Glu Val Glu Asp Leu Glu Tyr Ala Leu Asp Ser Ser Gly Arg 820 825 830				2793
ggt gtc aag tct ttg gag gaa att caa ctg gag ctg aac ttt ctg cag Gly Val Lys Ser Leu Glu Glu Ile Gln Leu Glu Leu Asn Phe Leu Gln 835 840 845 850				2841

aga aca agg gac aca ttg att gtc gaa gtg gat gat ctt aga gat caa Arg Thr Arg Asp Thr Leu Ile Val Glu Val Asp Asp Leu Arg Asp Gln 855 860 865	2889
cat aga atg cta aat gaa gat atg tca agt gct cag gtg aga tgg cac His Arg Met Leu Asn Glu Asp Met Ser Ser Ala Gln Val Arg Trp His 870 875 880	2937
aat gct cgg gaa gag aaa gtg aaa gct tct agc ata ttg gaa aga ttc Asn Ala Arg Glu Glu Lys Val Lys Ala Ser Ser Ile Leu Glu Arg Phe 885 890 895	2985
caa aaa tct gaa gag gaa ttg gtg ctt cta gct gag gaa aaa gaa caa Gln Lys Ser Glu Glu Leu Val Leu Leu Ala Glu Glu Lys Glu Gln 900 905 910	3033
ctg att gta gaa aag aag ctt tta gaa gag tct ctt gat cca ttg tcc Leu Ile Val Glu Lys Lys Leu Leu Glu Glu Ser Leu Asp Pro Leu Ser 915 920 925 930	3081
aaa gag aaa gag agc ttg ttg caa gag tat aat gct ttg aag caa aag Lys Glu Lys Glu Ser Leu Leu Gln Glu Tyr Asn Ala Leu Lys Gln Lys 935 940 945	3129
ctg gat gaa gag tat cat cag ctt gca gaa aga aaa agg gag ttc cag Leu Asp Glu Glu Tyr His Gln Leu Ala Glu Arg Lys Arg Glu Phe Gln 950 955 960	3177
caa gaa ctt gat gct ctt gga aga ctt aat atg aag ata aaa ggg tac Gln Glu Leu Asp Ala Leu Gly Arg Leu Asn Met Lys Ile Lys Gly Tyr 965 970 975	3225
ttg gat tcc aag aaa aac gaa aag ctt aag gaa ttg cag gga agg cat Leu Asp Ser Lys Lys Asn Glu Lys Leu Lys Glu Leu Gln Gly Arg His 980 985 990	3273
gtt ctt tgc cat tct cag tta cag agt tgc atg gca aaa cag caa aga Val Leu Cys His Ser Gln Leu Gln Ser Cys Met Ala Lys Gln Gln Arg 995 1000 1005 1010	3321
ata tca gct gag tta aac aag agc aaa gaa cta ctg cag ggc cag ggc Ile Ser Ala Glu Leu Asn Lys Ser Lys Glu Leu Leu Gln Gly Gln Gly 1015 1020 1025	3369
cag ttg aaa aga aac att gat gac aat ctc aag tac agg aaa aca aag Gln Leu Lys Arg Asn Ile Asp Asp Asn Leu Lys Tyr Arg Lys Thr Lys 1030 1035 1040	3417
gct gat gtg gaa caa ctt act cgt gat ata gaa tca ctt gaa gaa agg Ala Asp Val Glu Gln Leu Thr Arg Asp Ile Glu Ser Leu Glu Glu Arg 1045 1050 1055	3465
ctg ctt tca ata ggt agc ttg tct gct ata gaa gct gat ctg aaa cgc Leu Leu Ser Ile Gly Ser Leu Ser Ala Ile Glu Ala Asp Leu Lys Arg 1060 1065 1070	3513
cat tct caa gaa aaa gag agg ctt aat tca gaa ttt aac agg tgg caa His Ser Gln Glu Lys Glu Arg Leu Asn Ser Glu Phe Asn Arg Trp Gln 1075 1080 1085 1090	3561
gga aca ctt tct gtt tat caa agt aat tca aag cac aaa caa gag Gly Thr Leu Ser Val Tyr Gln Ser Asn Ile Ser Lys His Lys Gln Glu	3609

1095	1100	1105	
ctt aaa ctg tca cag tac aag gat atc gag aag cga tat act aat caa Leu Lys Leu Ser Gln Tyr Lys Asp Ile Glu Lys Arg Tyr Thr Asn Gln 1110	1115	1120	3657
ttt ctc cag ctt aag aca act gaa atg gca aac aag gac ttg gac aga Phe Leu Gln Leu Lys Thr Thr Glu Met Ala Asn Lys Asp Leu Asp Arg 1125	1130	1135	3705
tat tat act gct tta gac aag gct ctt atg cgg ttc cac agc atg aag Tyr Tyr Thr Ala Leu Asp Lys Ala Leu Met Arg Phe His Ser Met Lys 1140	1145	1150	3753
atg gag gag ata aat aaa ata atc aag gaa ctg tgg caa cag aca tac Met Glu Glu Ile Asn Lys Ile Ile Lys Glu Leu Trp Gln Gln Thr Tyr 1155	1160	1165	3801
aga ggc cag gat att gat tac ata agc ata aat tct gat tct gag ggt Arg Gly Gln Asp Ile Asp Tyr Ile Ser Ile Asn Ser Asp Ser Glu Gly 1175	1180	1185	3849
gct ggc act cga tca tac agc tac cgc gtt gtt atg caa act ggt gat Ala Gly Thr Arg Ser Tyr Ser Tyr Arg Val Val Met Gln Thr Gly Asp 1190	1195	1200	3897
gct gag ctg gaa atg cga ggg cgc tgc agt gct ggt cag aag gtt ctt Ala Glu Leu Glu Met Arg Gly Arg Cys Ser Ala Gly Gln Lys Val Leu 1205	1210	1215	3945
gct tct ctt ata atc aga cta gca ctt gcg gaa act ttc tgc ctg aac Ala Ser Leu Ile Ile Arg Leu Ala Leu Ala Glu Thr Phe Cys Leu Asn 1220	1225	1230	3993
tgc ggt ata ttg gct ttg gat gag cca act acg aat cta gat ggg cca Cys Gly Ile Leu Ala Leu Asp Glu Pro Thr Thr Asn Leu Asp Gly Pro 1235	1240	1245	4041
aat gca gag agt ctt gct gcg ctg ttg aga ata atg gaa gcc agg Asn Ala Glu Ser Leu Ala Ala Leu Leu Arg Ile Met Glu Ala Arg 1255	1260	1265	4089
aaa ggg cag gag aac ttc cag ttg att gta atc act cat gat gag aga Lys Gly Gln Glu Asn Phe Gln Leu Ile Val Ile Thr His Asp Glu Arg 1270	1275	1280	4137
ttt gcc cat ctt atc ggt caa agg cag ctt gct gag aag tac tat cga Phe Ala His Leu Ile Gly Gln Arg Gln Leu Ala Glu Lys Tyr Tyr Arg 1285	1290	1295	4185
gtc tcc aag gat gag aac cag cac agc ata att gaa tcc caa gag ata Val Ser Lys Asp Glu Asn Gln His Ser Ile Ile Glu Ser Gln Glu Ile 1300	1305	1310	4233
ttt gac taagggtgtt ctaggaggct gtagcacgca ctcgtttgct agtcgaatcc Phe Asp 1315			4289
agttaattta tgccaagtac tggtgccaga gcaatgttaa caagcttttag gaggctctgt tacgcgtta cgtcagttgc gtgaaaccta tccttcgttg ttgtatactt atttatctg gccagaggat ggaatgtgtg cactgggtga tggatgttc acgacatcaa tgaatgttc acaatctagc atcaaaaaaaa aaa			4349 4409 4469 4492

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 35 40 45
 Leu Ser Cys Thr Gly Glu Leu Pro Pro Asn Ser Arg Ser Gly His Thr
 50 55 60
 Phe Val His Asp Pro Lys Val Ala Gly Glu Thr Glu Thr Lys Gly Gln
 65 70 75 80
 Ile Lys Leu Arg Phe Lys Thr Ala Ala Gly Lys Asp Val Val Cys Ile
 85 90 95
 Arg Ser Phe Gln Leu Thr Gln Lys Ala Ser Lys Met Glu Phe Lys Ala
 100 105 110
 Ile Glu Ser Val Leu Gln Thr Ile Asn Pro His Thr Gly Glu Lys Val
 115 120 125
 Cys Leu Ser Tyr Arg Cys Ala Asp Met Asp Arg Glu Ile Pro Ala Leu
 130 135 140
 Met Gly Val Ser Lys Ala Val Leu Glu Asn Val Ile Phe Val His Gln
 145 150 155 160
 Asp Glu Ser Asn Trp Pro Leu Gln Asp Pro Ser Thr Leu Lys Lys Lys
 165 170 175
 Phe Asp Asp Ile Phe Ser Ala Thr Arg Tyr Thr Lys Ala Leu Glu Val
 180 185 190
 Ile Lys Lys Leu His Lys Asp Gln Met Gln Glu Ile Lys Thr Phe Arg
 195 200 205
 Leu Lys Leu Glu Asn Leu Gln Thr Val Lys Asp Gln Ala His Lys Leu
 210 215 220
 Arg Glu Asn Ile Ala Gln Asp Gln Glu Lys Ser Asp Ala Ser Lys Ser
 225 230 235 240
 Gln Met Glu Gln Leu Lys Glu Lys Ile Cys Gly Thr Glu Arg Glu Ile
 245 250 255
 Leu Gln Met Glu Thr Ser Leu Asp Glu Leu Arg Arg Leu Gln Gly Gln
 260 265 270
 Ile Asp Ile Lys Ala Thr Glu Arg Ser Thr Leu Leu Thr Gln Gln His
 275 280 285
 Glu Lys Leu Ala Ala Leu Ser Glu Glu Asn Glu Asp Thr Asp Glu Glu
 290 295 300
 Leu Met Glu Trp Gln Thr Lys Phe Glu Glu Arg Ile Ala Leu Leu Glu
 305 310 315 320
 Thr Lys Ile Ser Lys Leu Val Arg Asp Met Asp Asp Glu Ala Ser Tyr
 325 330 335
 Ser Ser Val Leu Ser Lys Gln Asn Ser Glu Leu Thr His Glu Ile Gly
 340 345 350
 Lys Leu Gln Ala Glu Ala Asp Ala His Leu Thr Met Lys His Glu Arg
 355 360 365
 Asp Ser Asp Ile Lys Asn Ile Cys Thr Lys His Asn Leu Gly Pro Val
 370 375 380
 Pro Glu His Pro Phe Thr Asn Asp Val Ala Met Asn Leu Thr Asn Arg
 385 390 395 400
 Ile Lys Ala Arg Leu Ser Ser Leu Glu Asn Asp Leu Leu Asp Lys Lys
 405 410 415
 Lys Ser Asn Glu Asp Gln Leu Asp Val Leu Trp Lys His Tyr Leu Lys
 420 425 430
 Ile Asn Ala Arg Tyr Ser Glu Val Asp Gly Gln Ile Gln Ser Lys Ile

435	440	445
Glu Ser Met Ser Gly Ile Leu Arg Arg Arg Lys Asp Lys	Glu Lys Glu	
450	455	460
Arg Asp Ala Ala Glu Val Glu Leu Ser Lys Phe Asn	Leu Ser Arg Ile	
465	470	475
Asp Glu Arg Glu Arg His Met Gln Ile Glu Val Glu Arg	Lys Thr Leu	
485	490	495
Ala Leu Gly Glu Arg Asp Tyr Asp Ser Ile Ile Ser Gln	Lys Arg Thr	
500	505	510
Glu Val Tyr Ser Leu Glu Gln Lys Ile Lys Val Leu	Leu Arg Glu Lys	
515	520	525
Asp Ile Ile Asn Arg Asn Ala Asp Glu Arg Val Lys	Leu Gly Leu Lys	
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Lys Asp Ala Leu Glu Ser Ser Lys Asp Lys	Leu Asn Glu Ile Val Asn	
545	550	555
Glu His Lys Asp Lys Ile Lys Lys Val Leu Arg Gly	Arg Asn Pro Phe	
565	570	575
Glu Lys Asp Met Lys Lys Glu Ile Asn Gln Ala Phe	Trp Pro Val Asp	
580	585	590
Lys Glu Tyr Asn Glu Leu Arg Ser Lys Ser Gln Glu	Ala Glu Gln Glu	
595	600	605
Leu Lys Phe Thr Gln Ser Lys Val Thr Asp Ala Arg	Glu Gln Leu Thr	
610	615	620
Lys Leu Arg Arg Asp Met Asp Ala Lys Arg Arg	Phe Leu Asp Ser Lys	
625	630	635
Leu Gln Ser Ile Leu Gln Ile Ser Ala Asn Val Asp	Met Phe Pro Lys	
645	650	655
Val Leu Gln Asp Ala Met Asn Lys Arg Asp Glu Gln	Lys Arg Leu Glu	
660	665	670
Asn Phe Ala Asn Gly Met Arg Glu Met Leu Ala Pro	Phe Glu His Leu	
675	680	685
Ala Arg Lys Asn His Val Cys Pro Cys Cys Glu Arg	Ala Phe Thr Pro	
690	695	700
Asp Glu Glu Asp Glu Phe Val Lys Lys Gln Arg Met	Gln Asn Ser Ser	
705	710	715
Thr Ala Glu Arg Ser Lys Ala Leu Ala Met Glu Ser	Ser Asn Ala Glu	
725	730	735
Ala Leu Phe Gln Gln Leu Asp Lys Leu Arg Thr Ile	Tyr Asp Ala Tyr	
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Val Lys Leu Val Glu Glu Thr Ile Pro Leu Ala Glu	Lys Asn Leu Asn	
755	760	765
Gln His Leu Ala Asp Glu Ser Gln Lys Ala Gln Ala	Phe Asp Asp Leu	
770	775	780
Leu Gly Val Leu Ala His Val Gln Met Asp Arg Asp	Ala Val Glu Ala	
785	790	795
Leu Leu Gln Pro Thr Asp Thr Ile Asp Arg His Val	His Glu Ile Gln	
805	810	815
Gln Leu Val Lys Glu Val Glu Asp Leu Glu Tyr Ala	Leu Asp Ser Ser	
820	825	830
Gly Arg Gly Val Lys Ser Leu Glu Glu Ile Gln Leu	Glu Leu Asn Phe	
835	840	845
Leu Gln Arg Thr Arg Asp Thr Leu Ile Val Glu Val	Asp Asp Leu Arg	
850	855	860
Asp Gln His Arg Met Leu Asn Glu Asp Met Ser Ser	Ala Gln Val Arg	
865	870	875
Trp His Asn Ala Arg Glu Glu Lys Val Lys Ala Ser	Ser Ile Leu Glu	
885	890	895
Arg Phe Gln Lys Ser Glu Glu Glu Leu Val Leu	Leu Ala Glu Glu Lys	
900	905	910
Glu Gln Leu Ile Val Glu Lys Lys Leu Leu Glu Glu	Ser Leu Asp Pro	
915	920	925
Leu Ser Lys Glu Lys Glu Ser Leu Leu Gln Glu	Tyr Asn Ala Leu Lys	

930	935	940	
Gln Lys Leu Asp Glu Glu Tyr His Gln Leu Ala Glu Arg Lys Arg Glu			
945	950	955	960
Phe Gln Gln Glu Leu Asp Ala Leu Gly Arg Leu Asn Met Lys Ile Lys			
965	970	975	
Gly Tyr Leu Asp Ser Lys Lys Asn Glu Lys Leu Lys Glu Leu Gln Gly			
980	985	990	
Arg His Val Leu Cys His Ser Gln Leu Gln Ser Cys Met Ala Lys Gln			
995	1000	1005	
Gln Arg Ile Ser Ala Glu Leu Asn Lys Ser Lys Glu Leu Leu Gln Gly			
1010	1015	1020	
Gln Gly Gln Leu Lys Arg Asn Ile Asp Asp Asn Leu Lys Tyr Arg Lys			
1025	1030	1035	1040
Thr Lys Ala Asp Val Glu Gln Leu Thr Arg Asp Ile Glu Ser Leu Glu			
1045	1050	1055	
Glu Arg Leu Leu Ser Ile Gly Ser Leu Ser Ala Ile Glu Ala Asp Leu			
1060	1065	1070	
Lys Arg His Ser Gln Glu Lys Glu Arg Leu Asn Ser Glu Phe Asn Arg			
1075	1080	1085	
Trp Gln Gly Thr Leu Ser Val Tyr Gln Ser Asn Ile Ser Lys His Lys			
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Gln Glu Leu Lys Leu Ser Gln Tyr Lys Asp Ile Glu Lys Arg Tyr Thr			
1105	1110	1115	1120
Asn Gln Phe Leu Gln Leu Lys Thr Thr Glu Met Ala Asn Lys Asp Leu			
1125	1130	1135	
Asp Arg Tyr Tyr Thr Ala Leu Asp Lys Ala Leu Met Arg Phe His Ser			
1140	1145	1150	
Met Lys Met Glu Glu Ile Asn Lys Ile Ile Lys Glu Leu Trp Gln Gln			
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Thr Tyr Arg Gly Gln Asp Ile Asp Tyr Ile Ser Ile Asn Ser Asp Ser			
1170	1175	1180	
Glu Gly Ala Gly Thr Arg Ser Tyr Ser Tyr Arg Val Val Met Gln Thr			
1185	1190	1195	1200
Gly Asp Ala Glu Leu Glu Met Arg Gly Arg Cys Ser Ala Gly Gln Lys			
1205	1210	1215	
Val Leu Ala Ser Leu Ile Ile Arg Leu Ala Leu Ala Glu Thr Phe Cys			
1220	1225	1230	
Leu Asn Cys Gly Ile Leu Ala Leu Asp Glu Pro Thr Thr Asn Leu Asp			
1235	1240	1245	
Gly Pro Asn Ala Glu Ser Leu Ala Ala Leu Leu Arg Ile Met Glu			
1250	1255	1260	
Ala Arg Lys Gly Gln Glu Asn Phe Gln Leu Ile Val Ile Thr His Asp			
1265	1270	1275	1280
Glu Arg Phe Ala His Leu Ile Gly Gln Arg Gln Leu Ala Glu Lys Tyr			
1285	1290	1295	
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<212> DNA

<213> Artificial Sequence

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<223> Designed oligonucleotide based upon an adaptor
used for cDNA library construction and poly(dT) to
remove clones which have a poly(A) tail but no
cDNA insert.

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36

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